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PESTICIDE PROGRAM

Guidelines for Registering Pesticides in United States

ENVIRONMENTAL PROTECTION AGENCY

[40 CFR Part 162]

[FRL 375–1; OPP–30004]

PESTICIDE PROGRAMS

Guidelines for Registering Pesticides in the United States

On October 16, 1974, the Environmental Protection Agency (EPA) published in the FEDERAL REGISTER (39 FR 36976) proposed regulations for the registration, re-registration and classification of pesticides under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended (7 U.S.C. 136 et seq.) In that publication, as well as in the FEDERAL REGISTER issued October 23, 1974 (39 FR 37670), notice was given that preliminary draft Registration Guidelines, §§ 162.40, 162.63–162.82, (hereafter referred to as "Guidelines") had been made available for public review at the Environmental Protection Agency's Headquarters in Washington, D.C. and at the Regional Offices, and that the Agency later would issue these Guidelines as formal proposals for comment.

The Guidelines were initially organized into 8 major parts, but have since been expanded with the addition of Section 162.83, *Packaging, storage and disposal.* Sections 162.40, 162.63–162.82 have been completed and are presented here for public comment. Section 162.41 *Registration procedures,* § 162.52, *Experimental use permits,* § 162.83 *Petitions for tolerances,* and § 162.94 *Packaging, storage and disposal* are not included at this time. Upon their completion, these sections will be issued as formal proposals for comment and will subsequently be added to the Guidelines.

Section 162.40 consists of introductory material describing the scope and the intent of the Guidelines. Section 162.63 offers guidance to the registrant on data required to substantiate proposed claims for product performance. Section 162.71 addresses the factors that must be considered by the applicant in the development of the product label. Section 162.77, *Chemistry,* relates to data necessary for the general evaluation of products and individual ingredients. Section 162.80 describes the data required of the applicant to evaluate product hazard to humans, domestic animals, fish and wildlife. In recognition of the fact that the nature of required data often depends upon specific product parameters, data are described as either unconditionally required, in which case it is required of all pesticides, or conditionally required, in which case the product parameters are specified.

This copy of the Guidelines constitutes the seventh version of the manuscript. Copies of drafts 1 (January 3, 1972) and 2 (May 1, 1972) were circulated, on a limited basis, to segments of the pesticide industry and to interested government agencies. Draft 3 (dated March 21, 1973) was submitted to some 200 reviewers representing other Agency programs, other Federal agencies, pesticide manufacturing groups, user groups, the National Agricultural Chemical Association (NACA), environmentalist groups, State Agricultural Experiment Station personnel, the academic community and scientific associations. Based on their reviews, draft 4 (November 30, 1973) was prepared. Many specific and general comments received on draft 4 were collated and individually assessed by joint *ad hoc* committees comprised of personnel from OPP and the Chairman of the Guidelines Committee of NACA. Draft 5 (October 1974) of the document underwent interagency review and reflects other agencies' comments. Draft 6 reflects further refinements of draft 5.

Significant comments received during the development of the document were limited to four main areas. One, comments were critical of apparent inflexibility of the Guidelines. Comments indicated the Guidelines were too dogmatic and suggested dropping the strong, hardline attitude for a more relaxed attitude offering examples of suggested methods rather than required methods. Two, some comments concerning genetic activity of pesticides could not be adequately included because the state of the art of genotoxic testing and evaluation is not sufficiently well developed. The Science Advisory Board of EPA has been asked to review this subject at the same time EPA is receiving public comment on these guidelines. As further acceptable and substantive information and test protocols (related especially to toxicological effects) become available, however, they will be incorporated into the Guidelines and the Appendices. Three, chemistry data requirements were subjected to considerable criticism. Chemistry data requirements are still stringent, but not as much as in the original document. Four, comments expressed difficulty with the organization of the original document. As a result of the comments, a careful editorial review was made in which the chapters were reorganized and new chapter headings were created.

OVERVIEW

This Agency is required to publish guidelines specifying the kinds of information which will be required to support the registration of a pesticide. Moreover, the Act requires the Administrator to register a pesticide if he determines that, when considered with any imposed restrictions:

(1) Its composition is such as to warrant the proposed claims for it;

(2) Its labeling and other material required to be submitted comply with the requirements of this Act;

(3) It will perform its intended function without unreasonable adverse effects on the environment; and

(4) When used in accordance with widespread and commonly recognized practice it will not generally cause unreasonable adverse effects on the environment.

1. SECTION 162.63. PRODUCT PERFORMANCE

The development of a substance or mixture of substances into a pesticide must include preliminary tests to evaluate its effectiveness and its usefulness. A product must, of course, be effective to be useful, and its usefulness is negated if, for example, it also kills desirable plants, causes harm to non-target species or has other deleterious effects. Effectiveness ascertains the product's ability to control the specified target pest or produce the specified plant action. Usefulness ascertains man's ability to apply the product according to the directions and cautions on the label without causing unreasonable adverse effects. Laboratory, greenhouse and/or small plot field testing are conducted to determine the inherent effectiveness of a substance against specific pest organisms. Once this is established, evidence of the effectiveness and usefulness of the proposed product is augmented through advanced large-scale laboratory and/or field-testing procedures which closely approach actual commercial use and which employ commercial application equipment.

The actual test procedures will vary according to the chemical characteristics, the type of formulation and packaging, the dosage rates or use dilutions, the methods and timing of application, and the target pest. Certain basic techniques are essential, however, as outlined under General Considerations in this section. This section also includes specific problems as discussed under Special Considerations. Because an acceptable test for one product or use may not be valid to support another product intended for the same use or for the same product intended for even a slightly different use, it is impossible to specify mandatory methods in this guide. Suggestions are provided in the Appendices, but the registrant or his testing agent must be responsible for the validity of the particular test or tests employed. The relationship of the product performance test results to label directions for use and limitations is presented in § 162.64, Label development.

2. SECTION 162.71. LABEL DEVELOPMENT

Clear and easily understood labeling is essential to the proper use of any pesticide. The Agency has a continuing interest in the improvement of the quality of pesticide labels. Section 162.10 of the regulations, dealing with the registration and classification of pesticides, treats in detail the labeling requirements of FIFRA, as amended, and attempts to improve the communicative value of labels and labeling by complete coverage of required warnings and precautionary statements, statement of use classification, labeling format, which must be considered in developing acceptable labeling.

Parameters which must be considered in developing acceptable labeling are treated in the Guidelines under subsections entitled General Comments and Specific Comments. The information contained under General Comments pertains to all pesticide products and provides guidance on acceptable claims, directions for use, limitations for use, and precautionary statements. Additional detailed information for specific types

of products is provided under the Specific Comment subsection.

3. SECTION 162.77. CHEMISTRY

The physical and chemical nature of a pesticide, its metabolites and degradation products are important determinants of potential hazard. This section is divided into two parts. The first, General Product Chemistry, relates the data necessary for the basic manufacturing process, information required for active and inert ingredients, assay and impurity assessment for technical products, information required for formulations, storage stability data, and residue information for pesticides on tobacco. The second part, Environmental Chemistry, discusses the following studies to measure the environmental impact of pesticides: pesticide fate and movement studies, pesticide residue studies, photodegradation, volatilization, and microbiological studies.

On June 23, 1970, the Agency notified persons responsible for Federal registration of pesticides of studies that were determined to be necessary to determine the impact of pesticides on the environment (PR Notice 70-50). These studies are designed to characterize the potential environmental significance of the various biological, chemical and physical processes that occur with the dissipation of pesticides. These studies are maintained in the Guidelines as the bases for many of the present test requirements. Studies called for, among other things, the rate of dissipation of the pesticide in soil, the mechanism of degradation of the pesticide residues, accumulation studies of the parent compound and principle metabolites in fish, rabbit, bird tissue, leaching studies, and studies to determine pesticide fate and movement in water. To these the Guidelines have added volatilization studies as a new environmental chemistry requirement designed to test pesticides for outdoor uses.

4. SECTION 162.80. HAZARD EVALUATION

Toxicological effects depend upon the chemical properties of the pesticide, the life stage of the exposed organisms, and the environmental conditions at the time of exposure. The toxic effects can be such that they may cause immediate death, reduced activity, paralysis, or other debilitations. Short-term acute effects may occur when a highly reactive compound acts upon a particular state of an animal or plant. In the case of long-term or chronic effects, subtle detrimental effects such as behavioral changes and induction of tumors may appear after a period of time.

Section 162.80 discusses the toxicological information necessary to assess toxicity to man and domestic animals, and fish and wildlife. It sets forth methods for collecting and reporting toxicity data which an applicant for registration or reregistration of a product must provide to the Agency pursuant to 40 CFR 162.8. This toxicity data enables the Agency:

(1) To determine whether registration should be approved and whether the pesticide product should be classified for general use or restricted use according to the criteria of 40 CFR 162.11;

(2) To determine whether the proposed labeling contains adequate precautions and warnings to protect pesticide users, exposed humans, domestic animals, fish and wildlife;

(3) To establish residue tolerances to protect consumers of exposed foods; and

(4) To determine conditions under which farm workers can reenter treated fields.

a. *Hazards to humans and domestic animals.* In the early 1950's toxicological requirements for registration of a pesticide was generally limited to an acute oral study (single dose) and a subacute study (30 to 90 day exposure). There was little concern at that time for the possible effects on people of long-term, low level exposure to pesticides. The situation is very different today. An increased awareness exists of the possible long-term impact of pesticides on man and the environment. Along with this increased public awareness has been the development of powerful analytical methods of detecting trace amounts of pesticide residues and of new test systems for evaluating the teratogenic, oncogenic, and mutagenic potential of chemicals.

Regulations issued under the amended FIFRA specify conditions under which an evaluation of the teratogenic, oncogenic, and mutagenic potential of chemicals. These conditions, which are detailed in the Guidelines, increase the number of pesticides that require such evaluation. However, in most cases information is required on an active ingredient basis and therefore testing does not have to be performed for each individual product for which an application for registration is submitted. In addition to the oncogenic study, a requirement is conditionally specified in the Guidelines for a two-year feeding study on appropriate test animals. The Agency recognizes that the two-year feeding study and the oncogenic study can be combined into a single test if correct protocols are observed. However, two rather than one test are specified to emphasize the Agency's requirement of well designed studies for oncogenic evaluation.

In the past if a pesticide received a negligible tolerance for food or feed use only subacute testing was required. These Guidelines, in conjunction with regulations issued under the amended Act, require the evaluation of oncogenic, chronic feeding and reproduction effects for all pesticides that have food or feed uses, unless specific exemptions from such requirements are given by the Agency. These requirements are prompted by the fact that even low pesticide residue levels in the human diet cannot, in general, be equated to toxicological insignificance.

Often conditionally required tests described in the Guidelines are acute and subacute inhalation tests, subacute dermal and oral tests, neurotoxicity tests, and metabolism and potentiation studies. In addition, required tests for all pesticide registrations include acute oral LD-50, acute dermal LD-50 and primary dermal irritation and primary eye irritation. The requirements for these acute and subacute tests are not changed by the Guidelines.

EPA published worker protection standards for agricultural pesticides on May 10, 1974. Included in these were label requirements specifying a permissible reentry data will be required for cholinesterase inhibiting pesticides. The Guidelines reflect this new data requirement.

b. *Hazards to fish and wildlife.* To reduce the possible adverse effects which might result from fish and wildlife being exposed to quantities of pesticides in the environment, data must be secured for pesticides that will be used outdoors or that may contaminate water or other environmental resources.

Acute and subacute toxicities are the initial criteria by which potential hazard to fish and wildlife is evaluated. To provide a more complete environmental evaluation, the Agency has added to the Guidelines two fundamental acute tests and one chronic residue study. The two acute tests are the avian acute oral LD-50 and the invertebrate acute toxicity. The avian acute oral had been required up until 1971 at which time the avian subacute dietary LC-50 replaced this test. It is now felt that the avian acute oral LD-50 data is important for pesticide registration and has been reincorporated as a basic test along with the avian subacute dietary LD-50, fish acute toxicity, and invertebrate acute toxicity. Invertebrate acute toxicity data are required for sensitive aquatic invertebrates. As a conditional test, subacute or chronic fish and/or invertebrate reproduction studies normally will be required if the pesticide is used in, or likely to contaminate, aquatic environments. In addition, other conditional tests for fish and wildlife include mammalian toxicity data as required for evaluating hazard to humans and domestic animals, acute toxicity data (LC-50, 96-hour protocol) for shrimp, crabs, and oyster larvae, satisfactory evidence that the pesticide will not cause unreasonable adverse effects on the flora and fauna in aquatic environments, reproduction studies on two species of birds, and any other toxicity data beyond those outlined in this section as requested on an individual basis.

LEGAL STATUS

Section 3(c)(2) of the FIFRA provides that the "Administrator shall publish guidelines specifying the kinds of information which will be required to support the registration of a pesticide and shall revise such guidelines from time to time." Furthermore, Agency is required to follow the provisions of the Administrative Procedure Act (APA). The APA (5 U.S.C. et seq.) defines "rule" to mean:

> * * * the whole or part of an agency statement of general or particular applicability of future effect designed to implement, interpret, or prescribe law or policy or describing the organization, procedure or practice requirements of an agency * * *

The issuance of the Guidelines is an implementation of the amended FIFRA, and, therefore, under this definition, constitutes rulemaking. The APA establishes two types of rulemaking procedures: "informal" and "formal" or adjudicatory rulemaking. Formal rulemaking procedures generally are required when the statute concerned expressly requires that rulemaking be conducted "on the record." FIFRA imposes no such requirements; therefore, informal rulemaking procedures are applicable to the Guidelines.

The Guidelines are to be used in conjunction with Title 40, Code of Federal Regulations, Part 162, Regulations for the Registration, Reregistration and Classification of Pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act. Other parts of 40 CFR that are pertinent to the registration of pesticides include Part 163, Certification of Usefulness of Pesticide Chemicals; Part 165, Regulations for the Acceptance of Certain Pesticides and Recommended Procedures for the Disposal and Storage of Pesticides and Pesticide Containers; Part 170, Worker Protection Standards for Agricultural Pesticides; and Part 180, Tolerances and Exemptions from Tolerances for Pesticide Chemicals in or on Raw Agricultural Commodities.

Purpose

The overall purpose of the Guidelines is to inform interested members of the general public of the factors entering into the pesticide registration process, to encourage and enhance the ability of the applicant for registration to present an adequate and completely documented application, and to assist the Agency in expediting the review procedure. Such guidance will reduce some of the uncertainty associated with achieving compliance with the requirements for registration. The Agency believes that the decisions, allowing for the range of variables involved in pesticide Guidelines as proposed will apply to the vast majority of registration decisions, allowing for the range of variables involved in pesticide registration.

Flexibility is a key element of the Guidelines; as new material is developed to suit changing technology, the existing document will be expanded and modified. Entire parts may be added or deleted as new policies and requirements are established, new test procedures introduced, or Agency procedures changed. The Agency may modify the data requirements in support of a particular application if additional data is required to assess the product. On the other hand, if an applicant questions the applicability of the Guidelines to his own situation, he may propose for approval an alternative approach better suited to his case. The Guidelines therefore are designed to serve the public interest without establishing requirements which are inflexible or inappropriate to particular registration actions.

Appendices to Guidelines

The significance and validity of information required by the Guidelines depend on the test procedures employed to develop it. Thus, the Guidelines are accompanied by the Appendices describing test procedures which have been determined to be adequate to provide data satisfying registration in the majority of cases. Such a comprehensive step has not been taken in the past with respect to Federal regulation of pesticides. The Appendices describe those test methods, procedures, or protocols related to developing registration data for: (1) Product efficacy; (2) environmental chemistry; and (3) hazards to humans and domestic animals and fish and wildlife. Because of the diversity of the materials, the information is presented in several formats:

(1) Bibliographic citations only, where material cited is available in open literature and the method or protocol is acceptable precisely as presented.

(2) Annotated bibliographic citations, where a modification, adaptation, or explanatory statement is necessary for acceptable data development for registration.

(3) Full text (with source credit) of unpublished methods or protocols of those that are not readily available.

(4) Full text of procedures compiled from several sources, authorities, or references.

The Appendices are divided into subject headings which correspond to the Guidelines. Some parts covering several types of pesticide products may be further divided alphabetically into subparts. Part IV. A, for example, will cover product performance related to aquatic pest control agents; Part IV. B, product performance related to aquatic pest control agents. Subject headings presently designated "reserved" will, as the Appendices are progressively developed, contain other material that may assist a registrant in compiling data or in completing application packages.

The Appendices contain examples of test procedures that are acceptable to the Agency. However, the Agency recognizes that applicants may be aware of other test procedures which are equally effective for particular purposes and that new procedures will be developed in the future. It also recognizes that the test methods described in the Appendices sometimes may not be well suited to the evaluation of certain products. Therefore, applicants for registration may be permitted to use procedures other than those set out in the Appendices, provided that the new methods do not detract from the intent and reliability of the Guidelines/Appendices. Similarly, applicants may be sometimes required to use other procedures if the protocols in the Appendices are not applicable. In all cases, the burden is on the applicant to exercise his best scientific judgment.

Means of Issuance

The Guidelines and Appendices will be published in the Federal Register in final form with such changes as the Agency deems warranted upon consideration of all comments. In order to facilitate their use, these documents and subsequent amendments will thereafter be published in a loose-leaf format, which will be available on a subscription basis from the Government Printing Office, Washington DC 20402. The loose-leaf format will allow quick and easy insertion and deletion of material.

The Guidelines and Appendices are not intended to be static. As new material is developed, the existing documents will be expanded and modified. Any major changes will be made in accordance with the informal rulemaking procedures of the APA. Changes to the Guidelines and Appendices which are routine or insignificant in nature or impact, and therefore unimportant to the industry and the public will be adopted without prior notice and opportunity to comment, pursuant to the provisions of the APA authorizing such procedures where the Agency finds that notice and comment are either impracticable, unnecessary, or contrary to the public interest.

Public Comment

Interested persons are invited to submit written comments with reference to this notice to the Federal Register Section, Technical Services Division (WH-569), Office of Pesticide Programs, Environmental Protection Agency, Room 401, East Tower, 401 M Street, SW., Washington, D.C. 20460. Three copies of the comments should be submitted to facilitate the work of the Environmental Protection Agency and others interested in inspecting the documents. The comments must be received on or before August 27, 1975, and should bear the identifying notation OPP-30004. All written comments filed pursuant to this notice will be available for public inspection in the Federal Register Section, Office of Pesticide Programs, from 8:30 a.m. to 4 p.m. Monday through Friday. A limited supply of copies are available at EPA Headquarters, Room 509 East Tower.

Dated: June 3, 1975.

Russell E. Train,
Administrator.

Part 162 of 40 CFR is amended by adding §§ 162.40 through 162.96 to read as follows:

General

Sec.

162.40 Scope and intent guidelines.

Registration Procedures

162.41–162.51 [Reserved]

Experimental Use Permits

162.52–162.62 [Reserved]

Product Performance

162.63 Overview.
162.64 General considerations.
162.65 Specific considerations: Antimicrobial agents.
162.66 Specific considerations: Aquatic pest control agents.
162.67 Specific considerations: Fungicides and nematicides.
162.68 Specific considerations: Herbicides, plant regulators, desiccants and defoliants.

Sec.
162.69 Specific considerations: Invertebrate control agents.
162.70 Specific considerations: Vertebrate control agents.

LABEL DEVELOPMENT

162.71 General comments.
162.72 Specific comments on antimicrobial agents.
162.73 Specific comments on aquatic pest control agents.
162.74 Specific comments on fungicides and nematicides.
162.75 Specific comments on invertebrate control agents.
162.76 Specific comments on vertebrate control agents.

CHEMISTRY

162.77 Overview.
162.78 General product chemistry.
162.79 Environmental chemistry.

HAZARD EVALUATION

162.80 Overview.
162.81 Hazards to humans and domestic animals.
162.82 Hazards to fish and wildlife.

PETITIONS FOR TOLERANCES

162.83–162.93 [Reserved]

PACKAGING, STORAGE AND DISPOSAL

162.94–162.96 [Reserved]

AUTHORITY: Sec. 3, Federal Insecticide, Fungicide, and Rodenticide Act, as amended (7 U.S.C. 136 et seq.).

GENERAL

§ 162.40 Scope and intent of guidelines.

(a) The legislative authority for pesticide registration is the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Pub. L. 88–305, as amended by the Federal Environmental Pesticide Control Act of 1972, Pub. L. 92–516. The Administrator of the Environmental Protection Agency is authorized by section 3(c)(5) of the amended FIFRA to register a pesticide if he determines that, when considered with any imposed restrictions:

(1) Its composition is such as to warrant the proposed claims for it;

(2) Its labeling and other material required to be submitted comply with the requirements of this Act;

(3) It will perform its intended function without unreasonable adverse effects on the environment; and

(4) When used in accordance with widespread and commonly recognized practice it will not generally cause unreasonable adverse effects on the environment.

If the Administrator determines that all of these requirements are satisfied, the registration application will be approved. To enable this determination, the applicant for registration must provide data in support of the application which is used to define its composition, to establish its efficacy, and to evaluate the hazard it poses.

(b) Section 3(c)(2) of the Act provides that the Administrator shall publish guidelines specifying the kinds of information which will be required to support the registration of a pesticide. This publication meets the requirement of section 3(c)(2). Its purpose is threefold: (1) To inform applicants of the correct procedures for registration and to explain the data requirements for registration set out in the regulations under section 3 of amended FIFRA (40 CFR Part 162); (2) to inform interested members of the general public of the factors used to make registration decisions; and (3) to assist registration officials in providing expeditious reviews by improving the completeness, quality and consistency of applications for registration.

(c) The validity of information submitted to meet data requirements depends on the test procedures employed and the expertise of the individuals performing the tests. Accordingly, these Guidelines include Appendices describing test procedures which have been determined to be adequate to develop data for registration purposes. Data developed in accord with these procedures will be acceptable; however, the Appendices do not prescribe inflexible registration requirements. The Agency recognizes that applicants may be aware of other test procedures which are equally effective for particular purposes, and that new procedures will be developed in the future. It also recognizes that the test methods described in the Appendices sometimes may not be well suited to the evaluation of particular products. Thus, modification of the specific procedures set out in the Appendices is permissible, providing the intent and reliability of the suggested methods are met. In cases where an applicant proposes to deviate from the procedures suggested in the Appendices, prior agreement with the Agency on the specific data development methods to be employed is strongly recommended.

(d) Neither the Guidelines themselves nor the Appendices are static documents. They will be expanded and revised periodically to reflect new scientific knowledge, new trends in pesticide development, and new Agency policies. All applicants, and new applicants in particular, are encouraged to consult with the Office of Pesticide Programs to resolve any questions relating to the Guidelines or data requirements. Any applicant who considers that certain provisions of the Guidelines may not be appropriate for a particular pesticide should submit a written statement of his position, and consult with the Office to determine what data are necessary in his particular case.

(e) Applicants are reminded that other statutes than FIFRA, as amended, affect the regulation of pesticides. A specific product proposed for registration under FIFRA may also be subject to provisions of the Federal Food, Drug, and Cosmetic Act; the Federal Water Pollution Control Act; the Federal Hazardous Substances Act; the Occupational Safety and Health Act; or the Poison Prevention Packaging Act. Specific references to appropriate statutes and their relevance to registration matters are made in other parts of these Guidelines.

(f) In addition to the internal efforts of the Office of Pesticide Programs, this publication reflects the critical reviews of experts from industry, academic communities, scientific associations, environmentalist groups, and other government agencies. The Office of Pesticide Programs is indeed indebted to these reviewers for their time and conscientiousness, but more important, for their specialized interests, acuities, and experience that have contributed heavily to making this a workable document.

REGISTRATION PROCEDURES

§§ 162.41–162.51 [Reserved]

EXPERIMENTAL USE PERMITS

§§ 162.52–162.62 [Reserved]

PRODUCT PERFORMANCE

§ 162.63 Overview.

(a) The development of a substance into a pesticide must include certain preliminary tests to evaluate its effectiveness and its usefulness. Laboratory, greenhouse, and/or small plot field-testing should be conducted to determine the inherent effectiveness of a substance against specific pest organisms. Once this is established, evidence of the effectiveness and usefulness of the proposed product must be further augmented through advanced large-scale laboratory and/or field-testing procedures which closely approach actual commercial use and which employ commercial application equipment. All tests should consider such factors as degree and duration of pest control, crop yield and quality, crop injury, flavor, animal acceptability, environmental injury and any others which confirm the safe, effective use of the product.

(b) The terms effectiveness and usefulness should be distinguished. Effectiveness refers to a product's ability to actually control the specified target pest or produce the specified plant action; usefulness refers to man's ability to apply the product according to the directions and cautions on the label without causing unreasonable adverse effects on himself, his environment, or his food. A product must, of course, be effective to be useful, but its usefulness is negated if, for example, it also kills desirable plants, causes harm to beneficial species, damages surfaces to which it is applied, or has other deleterious side effects.

(c) The actual test procedures will vary according to the characteristics of the chemical, the type of formulation, the target pest, the use patterns, the methods and timing of application, but certain basic techniques are essential, as outlined under General Considerations in this part. Specific problems are discussed under Special Considerations, also in this part. The relationship of test results to label directions for use and limitations is presented in Part V. Label Development. Because an acceptable test for one product or use may not be valid to support another product intended for the same use or for the same product intended for even a slightly different use, it is impossible to specify mandatory methods in this guide. Suggestions are provided in the Appendix below, but the registrant or his testing agent must be responsible for the validity of the particular test or tests employed.

(d) Testimonials or letters of recommendation are unacceptable in support of effectiveness claims. However, statements of opinion from individual cooperators regarding the efficacy and limitations of a particular product are of value when submitted in conjunction with test reports.

§ 162.64 General considerations.

(a) *Product parameters.* (1) *Formulation and packaging.* Several factors associated with formulating and packaging a pesticide influence the effectiveness of the active ingredient. The chemical and physical compatibilities of formulated pesticides, which include mixtures of pesticides and mixtures of pesticides with diluents, emulsifiers, fertilizers, solvents, spreaders, and stickers, must be evaluated. Formulated products proved effective in laboratory tests are sometimes ineffective as presented to the user for a variety of reasons, including short shelf life, chemical reaction with the repackaging component, or failure of the spray device to function properly. Or a product initially effective at the user level may suddenly become ineffective because of a change in the can liner coating, product emulsifier, solvent or other component used. Mixtures of 2 or more pesticides into a single formulation may react chemically, be physically incompatible (producing a useless product), or be mutually antagonistic or synergistic for effectiveness. For these reasons, final tests to support the effectiveness and usefulness of a product must be conducted with the formulation for which the registrant is applying for registration, and sometimes—especially in the case of pesticides marketed in cans—with the same packaging to be used commercially.

(2) *Dosage rates or use dilutions.* Data should indicate the minimum effective dosage and dosage range and demonstrate an adequate safety margin between the maximum label dosage and injury. Dosages should be reported in one or more of the following terms:

(i) Amount per unit of surface area.

(ii) Amount per unit volume of solvent or diluent.

(iii) Amount per unit volume or weight of commodity.

(iv) Amount per unit volume of space.

(v) Amount per unit volume or area; depth (acre-foot or hectare-meter).

(vi) Amount per linear distance of crop row.

(vii) Amount per animal.

(viii) Amount per unit weight of animals.

(ix) The length of time of spraying and the distance from the surface (for certain pressurized products).

(3) *Application techniques.* Each test report must clearly describe all test conditions that may be needed for proper evaluation. Pertinent topics may include the following:

(i) The type of application for each test, such as ultralow volume (ULV), low volume (LV) or conventional full coverage spray. ULV refers to a total volume of ½ gal, or less, of undiluted spray per acre. LV applies when the volume of spray per acre is more than ½ gal but not greater than 3 gal. When a product is diluted before or during application, the report must specify the quantities and type of each diluent. More specific criteria may be developed, directed toward the unique characteristics of certain classes of pesticides, as necessary.

(ii) The equipment used, such as mist blowers, hydraulic ground sprayers, backpack sprayers, dusters, soil injectors or incorporators, and aircraft (fixed wing or helicopter) and aircraft systems.

(iii) The method of application. Descriptions of soil injection applications should include such information as chisel spacing and depth of injection; soil surface applications should indicate depth and method of incorporation, if any; row treatments should indicate in-furrow, band-over-row or sidedressing application. Such terms as scrubbing, wiping, rinsing, soaking, flooding, and mopping can describe some methods of application.

(iv) The timing of applications. Test reports should specify the time at which treatment was begun and the interval, in days, between succeeding applications, in terms of one or more of the following:

(A) Dates.

(B) Number of days before or after planting, emergence, or harvest.

(C) The stage of growth of the crop.

(D) The stage of growth or expected appearance of the pest.

(v) Site of application. Contact pesticides must be applied to the pests themselves, whereas residual type products are applied to surfaces which will in turn be contacted by pests. The specific site of application required for effective use of residual pesticides may be related to feeding or other habits of insects and other animals, or the location of infection or contamination by fungi and bacteria. For example, dogs establish urinary scent posts, and repellents must be applied to these areas to be effective. Or as another example, because mites feed on the lower sides of plant leaves, nonsystemic miticides must be applied to these undersurfaces. The site of application may also be directly related to the mode of action of the pesticide. A systemic insecticide may be applied to foliage and be transported to other parts of the plant where it will control feeding insects. A plant growth regulator may be applied to the foliage and travel to the part of the plant where biological control occurs. A granular herbicide may be applied to the ground, be absorbed by the roots and be transported into the stem or foliage where it exerts its biological action.

(vi) Tank mixes and serial applications. Directions for use which imply or recommend that products be either mixed in the spray tank before application or applied in sequence must have registered directions, limitations and precautions appropriate for such intended uses.

(A) Tank mixtures may include either a pesticide product and an adjuvant or 2 or more pesticide products that are applied simultaneously by mixing them in a spray tank. The purpose of tank mixing is to improve the economy of application and, at the same time, to broaden the spectrum of pest species to be controlled; to improve the level of control; to reduce dosages of individual components; or to increase the duration of control. Directions for tank mixing of products must be supported by adequate performance data on the effectiveness and usefulness of the proposed mixture at the dosage rate(s) specified for each pest indicated. (See also chemistry and hazard evaluation data requirements.) The components of a pesticide mixture must be physically, biologically and chemically compatible; and determination of interaction is an essential part of the evaluation of mixtures. No method is presently available to predict possible interactions without actual testing. A combination of analysis of variance, a method for multiple range tests, and a method of determining synergistic and antagonistic responses can identify significant interactions, relate results from one treatment to another, and provide an order of magnitude of each result.

(B) Serial application is the use of one pesticide immediately or shortly after the use of another, so that the target area (usually soil) will contain both pesticides at the same time. The registrant may find it to his advantage, where tank mixing is impractical, to specifically direct serial applications on product labels. These directions must be supported by performance data on each product to be applied in serial applications.

(b) *Experimental design.* Experimental techniques should be performed with sufficient precision to make the results obtained convincing and adequate for registration purposes. Separate tests are usually necessary to evaluate product performance against each pest under each set of variables, or use conditions. Occasionally 2 or more pests may be present on or in the same target area and their control can be evaluated simultaneously. Pertinent test variables can include some of those discussed below.

(1) *Climatic factors.* The testing schedule should be designed to permit evaluations of the effectiveness of pesticides used under different environmental conditions (arid versus moderate and high precipitation, cool versus warm and hot temperatures, normally cloudy versus normally sunny conditions, and low versus high humidity or dew formation), if these are relevant. Important abnormal conditions may occur within a given area which cannot be considered in the test design but which can markedly affect test results. Any such effects observed should be reported in the conclusions.

(2) *Edaphic (Soil) Factors.* A number of variables relating to the soil may be pertinent in evaluating a pesticide product. Several of these, such as soil temperature, texture, fertility, organic matter content, tillage practices and irrigation and rotation schedules, measurably influence pesticide performance within a geographic area. Accordingly, field-tests should be designed on a factorial or other reasonable basis to include pertinent parameters. Safety to crops planted in the treated area the same season, and in following seasons,

must be demonstrated. Residue data also will be required for soil pesticides used in conjunction with food or feed crops planted just before, during, or after treatment. (See Chemistry.)

(3) *Geographic distribution of tests.* If products are to be marketed on a nationwide basis, they will be subjected to the impact of a number of differences in climate, soil texture, variety, strain and density of the pest, variety of crop, etc. Tests, then, must be conducted in each major geographic area in which a crop and/or pest are of economic importance. If the pest control programs are of local importance, the data should be obtained only from those local or regional areas concerned. If a crop is grown only in a particular territory or region of the world, outside the United States, adequate test reports will be accepted from that territory or region.

(4) *Product application.* If more than one method of application is to be employed, such as air and ground, experiments must be designed to obtain the required data for each method.

(i) Information detailing the type of application as row (in-furrow, band or sidedressing) or broadcast must accompany the test report data. Pesticides applied as row treatments cannot be accurately reported on a quantity-per-acre basis without also reporting the distance between rows and the width of the treated band.

(i) An increase in the amount of the product applied may result in illegal residues, reduced yield or other adverse reactions. A decrease in the amount of the product applied as a result of decreasing the distance between rows may lower the effectiveness of the product.

(5) *Statistical procedures.* Statistical procedures are usually necessary in evaluating product performance, and the experimenter should either be well-informed or have competent advice in experimental design, sampling procedures, and statistical methods of analysis. The test should be designed so that such analyses can be made if required. The direct comparison of group means of treated and untreated checkplots may be sufficient to show the effectiveness of a product. More extensive analysis will be required if the difference between means is small or if 2 or more variable factors or their interactions are being evaluated.

(i) In effectiveness testing, the probability that a particular treatment will provide effective pest control is usually measured as treated versus untreated check. Untreated checks, however, are not always practical, particularly when testing on very large areas against motile pests or in motile substrates: For example, aircraft applications for insect control, certain field-tests with vertebrate pest control agents, or herbicides applied to moving bodies of water. When they are impractical, a product must be tested against some other base, such as another product of known efficacy used as a reference standard, or conditions measured before the start of the test.

(ii) Small plots on vegetable and forage crops, such as 50 ft of row, and singlebranch or single-tree plots on fruits may provide precise results but are rarely sufficient to support valid conclusions about product performance. Plots should be large enough to reflect actual commercial use conditions, yet small enough not to impose an economic hardship on the registrant. Such factors as the crop grown, equipment used, expected incidence of the pest, need for residue samples, yield data, and quality studies often dictate the size of field plots.

(iii) The number of plot replicates should be large enough to furnish a confidence level of 95 percent. Generally, the smaller the size of the individual plots, the larger the number of replicates required.

(iv) Checks and controls should represent all of the variables within the test except the one being investigated. For instance, in testing the effectiveness of a product in controlling a pest on a crop, the test design should include areas of the field manipulated exactly as all other areas of that field except for the application of the test product. Thus, a baseline of no effect on a pest can be established with which to compare the effect of various dosage rates of the product.

(v) To be representative of a population, sampling procedures must ensure that the sample reflects all characteristics of the population. The size of samples required for reliable estimates will vary with the effect to be characterized. For example, entire plots may need to be harvested for accurate yield estimates, whereas representative portions of each plot will probably be sufficient for measuring pest control.

(c) *Effectiveness and usefulness.* The primary parameter to be judged is that of the action of the product in preventing, destroying, repelling or mitigating a pest; accelerating or retarding the rate of growth or otherwise altering the behavior of plants; defoliating plants or artificially accelerating the drying of plant tissue. The experiment is largely designed to evaluate this effectiveness, but it can also be designed to obtain information on usefulness in terms of adverse effects noted. Registration of a pesticide product is dependent upon its lack of unreasonable adverse effects on man or his environment. Most unfavorable effects can be avoided by providing proper directions for use and caution statements, but the possibility of such effects must be recognized. It is impossible to list all of the hazards which may arise, but some examples are noted below and others are given in Hazard Evaluation.

(1) Phytotoxicity may result from treatment. A good test design will provide for dosages higher than necessary for pest control to allow an estimate of the margin of safety between effective pesticide levels and those which injure the crop. Dosages of 2 to 4X the estimated maximum for effectiveness (both in concentration and total amount applied per unit area) are usually sufficient, but dosages greater than 6X may be necessary for mist blower or aircraft application. Phytotoxicity is usually measured in terms of chlorosis, malformed plant parts, leaf burning, plant wilting and death. Some injury can often be tolerated; plants may recover or the extent of injury may not be of economic or aesthetic importance—but it should be noted. Pesticide products and their metabolites whose phytotoxicity or plant residue data indicate the necessity for rotational restrictions beyond 2 normal potential plants (18 mo) are usually considered prohibitively persistent for issuance of a registration for which a tolerance is required.

(2) Yield determinations will require advanced planning in the test design. Pesticide treatment may reduce yield below a tolerable level or so alter the normal ripening process that economic problems arise in harvesting. But pesticide applications also frequently result in increased yield and precise data on such benefits should be submitted.

(3) Quality may be improved or severely impaired by the application of pesticides. Taste panel tests, color determinations, blemish counts, livestock palatability trials, etc., can be incorporated into the experimental design for food or feed crop uses. Test designs also can be developed to evaluate adverse pesticide effects on materials and surfaces. Fabrics, for example, may be discolored, stained, tenderized or the hand may be changed; leather may become brittle; or the bonding capacity of paint to surfaces may be destroyed.

(d) *Reporting data.* (1) Systematic and complete reporting and assembly of data from individual plot tests and field-tests to support label claims for performance of a product or mixture is essential for efficient and effective review.

(2) All details of the tests should be reported, with particular emphasis on those variables incorporated on the label directions, limitations and precautions. Such details include:

(i) Crop (varieties) or site treated
(ii) Location of the tests
(iii) Plot size
(iv) Number of replications
(v) Soil type and organic matter
(vi) Formulation used
(vii) Rate of application
(viii) Finished spray volume per acre
(ix) Time of application in terms of crop stage
(x) Stage of weed pests at time of application
(xi) Method of application
(xii) Pertinent comments regarding unusual test conditions or performance
(xiii) Interval between treatment and observation
(xiv) Interval between observations
(xv) Yields
(xvi) Time of harvest
(xvii) Percent control of specific pests compared to untreated controls and the commercial standard
(xviii) A rating scale (or percent) showing performance related to commercial acceptability and type of efficacy and/or injury noted.

(3) Considerations for assembling the reported efficacy data to expedite review should include:

(i) Index the data reports according to each claim on the label, such as rates, formulations, times and methods of application, specific weed response, crop response. Use numbered tabs to separate individual reports.

(ii) Prepare tabular summaries of the data where each horizontal line is equivalent to a test and the columns are label claims. Organize these summaries according to the test locations (States or geographic areas).

(iii) Summarize and draw conclusions related to label claims. Include analyses and evaluations.

(iv) If any data are included from the testing of formulations other than the one proposed for registration, they should be presented and summarized separately.

(4) A brief description noting if the product is translocated or acts on contact, and other general descriptions, would be helpful as available.

§ 162.65 Specific considerations: Antimicrobial agents.

(a) The term antimicrobials as used in this section includes all preparations intended for preventing, destroying or mitigating any bacteria, pathogenic fungi or viruses in any environment except (1) those on or in living man or other animals; (2) those on or in living plants; and (3) those on or in processed food, beverages, or pharmaceuticals. Antimicrobials include sterilizers, sporicides, disinfectants, virucides, sanitizers, bacteriostats, and fungicides and fungistats used against fungi that are pathogenic for man or other animals. (See also Specific Considerations: Fungicides.)

(b) In the development of antimicrobial pesticides, the manufacturer usually knows (1) the types of claims he wishes to make for his product; (2) where he intends for the product to be used; and (3) the level of activity he expects his product to possess. He is usually concerned about the performance data which will be required by the Agency for registering such a product. Since the extent of testing required by the Agency varies with each of the factors mentioned above, it is difficult to separate performance data requirements from these factors, and to speak of product performance in general terms.

(c) *Definitions of performance levels.* (1) Because of the variety of microorganisms to be controlled and the different claims and many use patterns of products, it is important that terminology and definitions be clearly understood by the registrant. A program for evaluating label claims must be based on uniform definitions and a common understanding of words and terms.

(2) According to Webster's Dictionary (unabridged), a disinfectant is an agent that frees from infection; especially: A chemical that destroys vegetative forms of harmful microorganisms but not ordinarily bacterial spores—used especially of substances suitable for application to inanimate objects.

(3) Disinfection connotes the combating of an infection, and infectious processes generally involve living plants, animals or other biological entities. Therefore, in the administration of FIFRA, as amended, environmental surfaces are deemed to be contaminated, not infected. Disinfectants are considered only in relation to the inanimate environment and refer to products that kill the growing forms, but not necessarily the resistant spore forms, of microorganisms. Where the intended use is specifically against a spore-forming microorganism, the spores also would have to be killed. Similarly, a disinfectant recommended for use specifically against a virus would have to inactivate the virus irreversibly. The term disinfectant implies a degree of specificity in that proper use is contingent on the purpose for which it is employed and on the type of microorganism which must be destroyed. A disinfectant is used where the complete elimination of an infectious or other undesirable microorganism is intended or required. The terms germicide, bactericide, germicidal and bactericidal are synonyms that can be used instead of disinfectant or disinfect, as defined under FIFRA, as amended. A germicide, bactericide, virucide, fungicide or sporicide is a product that is cidal or lethal to specific kinds of microorganisms, referring, respectively, to germs, bacteria, viruses, pathogenic fungi or spores.

(4) A virucide (viricide), according to Webster's Dictionary (unabridged) is a physical or chemical agent that destroys or inactivates viruses. The unqualified use of this term represents the product as cidal (lethal) against all viruses. For registration purposes, virucidal claims must be qualified; that is, specific viruses that have been shown by current laboratory methods to be irreversibly inactivated by the product must be named on the label.

(5) A bacteriostat is an agent that inhibits the growth of bacteria in the presence of moisture and, in so doing, may or may not affect the viability of the bacteria. A fungistat is a chemical agent acting similarly on pathogenic fungi. These microorganisms, when transferred to a different environment, may still be capable of growth and reproduction. Since pathogens in a state of chemical stasis are capable of initiating infections in living animals, bacteriostatic or fungistatic treatments cannot be recommended or used as replacements for disinfecting processes. See specific comments on fungicides and nematicides for a definition of the term fungicide in reference to nonpathogenic fungi.

(6) A sanitizer is an agent that reduces microbial contaminants to safe levels as determined by Public Health Ordinances or that reduces the microbial population by significant numbers where public health requirements have not been established. Sanitizers meeting Public Health Ordinance requirements are generally used on food contact surfaces and are recognized as sanitizing rinses. They are not disinfectant, germicidal or bactericidal rinses. The intent of use as a sanitizer or the specific claim to sanitize would identify a product as a pesticide. The term sanitary appearing on a label is identified as a state of cleanliness and does not carry with it connotations of pesticidal benefits. A detergent-sanitizer is a product that possesses the property of a cleaner and a sanitizer. It is considered as a representation of value against bacteria greater than that provided by an ordinary soap or an ordinary detergent. Likewise, a germicidal-detergent by these basic definitions would have to possess the properties of both a disinfectant and a cleaner.

(7) An antiseptic is an agent that opposes sepsis, putrefaction or decay by preventing or arresting the growth of microorganisms. The word antiseptic is restricted to applications on or in the living body of man or other animals. Products recommended for skin disinfection, hand sanitization, teat dips, udder washes and applications on farrowing sows are treatments on the living body of man or animals and are not subject to regulation under FIFRA, as amended. Such applications fall under the purview of the Federal Food, Drug, and Cosmetic Act. Concurrent jurisdiction is exercised by the Environmental Protection Agency and the Food and Drug Administration over products identified both as pesticides and drugs.

(8) Deodorizers are chemical agents of 2 basic types: (i) Those that prevent the formation of bacterial odors by acting upon microorganisms which produce them, and (ii) those that mask, chemically destroy or neutralize odors. Only those deodorizers that are described as type (i) are subject to regulation under the Act.

(9) To sterilize means to destroy all living microorganisms. A sterilizer is an agent or a device that will destroy or eliminate all forms of life, including all forms of vegetative bacteria, bacterial spores, fungi and viruses. This is an absolute term; qualified terms such as practically sterile and commercially sterile are not considered acceptable.

(d) *Products for Use on Hard Surfaces.*—(1) *Disinfectant.* When a disinfectant is recommended for use in areas where effectiveness against Gram-negative enteric bacteria is of primary importance, and lack of effectiveness against other species may not be a critical factor (for example, toilet bowls and urinals), only minimal claims, such as germicide, disinfectant, or bactericide are permitted on the label. A disclaimer of effectiveness against Gram-positive bacteria may be required.

(i) Minimal disinfectant activity must be substantiated with efficacy data derived from either the Association of Official Analytical Chemist's (AOAC) Use Dilution Method for water-soluble powders and liquid products or the AOAC Germicidal Spray Products Method[1] for spray products. Sixty replicates are required on each of 3 samples, representing 3 different batches, tested against Salmonella choleraesuis. One of these

[1] Complete citations for official test methods suggested in this section are included in a list of references in Appendix Part IV. Tentative test methods, suggested protocols, or test methods that require some modification for registration testing are entered separately in Appendix Part IV and cited individually in this section.

batches, aged to represent a 60-day shelf life stability study, must also be tested with 30 replicates on duplicate samples against S. choleraesuis.

(ii) When a disinfectant is recommended for general use in any area (except medical areas), or if it is represented as having a broad spectrum of activity, more extensive testing is required. In addition to the 60 replicates on each of 3 samples, representing 3 different batches, tested against S. Choleraesuis, 30 replicates on each of these 3 samples must be tested against Staphylococcus aureus and one other pathogen. One of these batches, aged to represent a 60-day shelf life stability study, must also be tested with 30 replicates on duplicate samples against S. choleraesuis. (Employ AOAC Use Dilution or Germicidal Spray Products Method.)

(iii) When a disinfectant is recommended for use in hospitals, clinics, dental offices, etc., 60 replicates are required on each of 3 samples, representing 3 different batches, tested against S. aureus. Thirty replicates on each of these 3 samples must also be tested against S. choleraesuis and Pseudomonas aeruginosa. One of these batches, aged to represent a 60-day shelf life stability study, must be further tested with 30 replicates on duplicate samples against S. aureus. (Employ AOAC Use Dilution or Germicidal Spray Products Method.)

(2) *Phenol Coefficients.* Any phenol coefficient claim requires testing by the AOAC Phenol Coefficient Method from duplicate samples of one batch against Salmonella typhosa. If a phenol coefficient is declared against any other bacterium, the test also must be run against each bacterium named. The AOAC Use Dilution Test must be employed as the confirmatory procedure for determining the disinfecting efficacy of a product and the basis for recommending an effective use dilution.

(3) *Pathogenic Fungi.* Claims against specific pathogenic fungi must be supported with efficacy data derived from duplicate samples of one batch using the AOAC Fungicidal Test.

(4) *Mycobacterium tuberculosis.* Claims against M. tuberculosis must be substantiated with efficacy data derived from the AOAC Tuberculocidal Activity Method on one batch of a liquid product under test. If the product is a spray, the procedure must be modified to conform with the AOAC Germicidal Spray Product Method using the media, microorganisms, etc., described in the AOAC Tuberculocidal Activity Method.

(5) *Other Microorganisms.* Claims for effectiveness against specific microorganisms other than those named in the AOAC Use Dilution Method or the AOAC Germicidal Spray Products Method can be accepted only when supported by efficacy data derived by the appropriate method on 10 replicates of one batch against each microorganism claimed.

(6) *Sporicides.* All sporicidal claims must be substantiated with efficacy data derived from the AOAC Sporicidal Test. The test requires 30 replicates on each of 2 kinds of carriers (porcelain penicylinders and surgical silk suture loops) against spores of both *Bacillus subtilis* and *Clostridium sporogenes* on 3 different batches (120 carriers per batch or total of 360 carriers). Thirty carriers of each type must also be tested against spores of either microorganism on duplicate samples of one of the 3 batches aged to represent a 60-day shelf life stability (total of 120 carriers for the stability test). Added claims for effectiveness against spore forming organisms other than *B. subtilis* and *C. sporogenes* must be substantiated with data derived from 30 replicates (porcelain penicylinders) on one batch.

(7) *Sterilizers.* The AOAC Sporicidal Test is the basis for substantiating sterilizing claims. In addition to the basic data (360 carriers) required for sporicidal claims, 30 carriers of each type must be tested against spores of *B. subtilis* and *C. sporogenes* on duplicate samples of one batch, aged to represent a 60-day shelf life stability study (total of 240 carriers plus 360 carriers equals 600 carriers). No growth is acceptable in any of the subculture or resubculture tubes from the 600 carriers. Any pesticide which is recommended for use as a sterlizing agent in a specific device must be tested by the AOAC Sporicidal Test in the device intended for use. The test procedure may be modified to allow for valid evaluation in the specific device in which the pesticide is tested.

(8) *Virucides.* Until an official method is available, any virological technique recognized as technically sound can be used to develop data to support virucidal claims. The technique employed must simulate actual in-use conditions insofar as possible. See Appendix Part IV.A for a suggested test method, Methods for Evaluating the Virucidal Activity of Disinfectants.

(9) *Sanitizers (Nonfood Contact Surfaces).* Many household products are detergent-sanitizer or detergent-disinfectant formulations. These products must be reasonably effective as cleaners and also fulfill the requirements for a sanitizer or disinfectant, as the case may be. No standard AOAC method exists for testing the efficacy of abrasive powders, foams or creams; however, a modification of the AOAC Use-Dilution Method is suggested in Appendix Part IV.A. Other products for household or institutional use bearing sanitizing claims for nonfood contact surfaces must be evaluated by the procedure outlined in Appendix Part IV.A.

(10) *Sanitizing Rinses (Food Contact Surfaces).* (i) Sanitizing rinse claims for iodophors, mixed halides and chlorine-bearing chemicals must be substantiated with efficacy data derived from the AOAC Available Chlorine Germicidal Equivalent Concentration Method. Data from one test on each of 3 samples representing 3 different batches tested against *S. typhosa* are required. Test results must show product concentrations equivalent in activity to 50, 100 and 200 ppm of available chlorine (reference standard sodium hypochlorite). Data from duplicate samples of one of these batches, representing a 60-day shelf life stability study, showing product concentrations equivalent in activity to the 3 reference standards, must be furnished. Chemical analyses may be substituted for biological testing on the 60-day sample.

(ii) Sanitizing rinse claims for quaternary ammonium compounds, chlorinated trisodium phosphate and anionic detergent-acid formulations must be substantiated with efficacy data derived from the AOAC Germidical and Detergent Sanitizers Method. Data from one test on each of 3 different batches against both *Escherichia coli* and *S. aureus* are required, along with data from duplicate samples of one of these batches representing a 60-day shelf life stability study against *E. coli*. When claims for the effectiveness of the product in hard water are made, data derived from tests on duplicate samples of one batch against both *E. coli* and *S. aureus* are required at the hard water tolerance claimed.

(iii) In testing the efficacy of detergent sanitizers, the AOAC standardized tests may have to be modified to include appropriate neutralizers that are not specified in the methods. In testing chlorinated trisodium phosphate, for example, sodium thiosulfate is required to neutralize the residual chlorine.

(11) *Floor waxes.* In general, claims are restricted to a bacteriostatic level of efficacy after application on flooring. Tests on representative surfaces coated with the wax are carried out in seeded agar or by an agar overlay technique. Since there is no recognized standard test for demonstrating efficacy and practical value of antibacterial floor waxes, it is suggested that written protocols of proposed tests be provided for review before the initiation of the tests.

(e) *Products for use on fabrics and textiles*—(1) *Laundry additives.* (i) Residual bacteriostatic claims for laundry additives must be substantiated with efficacy data derived from the AOAC Bacteriostatic Activity of Laundry Additives Method. Three samples representing 3 product batches must be tested against S. aureus and Klebsiella pneumoniae (aberrant, ATCC 4352) using 5, 1-sq in. treated swatches on seeded agar. Duplicate samples of one batch representing 60-day shelf life stability must be tested with 5 replicate fabric swatches on agar seeded with S. aureus.

(ii) Residual self-sanitizing claims should be substantiated by the suggested test method outlined in Appendix Part IV.A. Reasonable modification of the method or alternate test methods may be accepted: *Provided,* That they are submitted for review before testing.

(iii) For products bearing sanitizing claims, the Petrocci and Clarke Method (Appendix Part IV.A) may be employed to provide efficacy data, since no official test method is available. The test organisms are K. pneumoniae (ATCC 4352 or ATCC 13906) and S. aureus (ATCC 6558). Tests may also be conducted in a washing machine. General protocols, propagation of cultures, fabric-to-water ratios, test materials, water temperatures and subculture media must be the same as those specified in the Petrocci and Clarke method. Tests must be con-

ducted with 3 samples representing 3 product batches; each sample must be tested with 3 replicate fabric swatches against the 2 test bacteria. One sample representing a 60-day shelf life storage must be tested in duplicate against S. aureus. A 99.9 percent reduction in bacteria over the control count must be demonstrated within 10 minutes.

(iv) The Petrocci and Clarke method is also employed to support disinfecting claims, and, again, the tests are conducted with 3 samples representing 3 product batches. Each sample must be tested with 30 replicate fabric swatches against the 2 test bacteria. One sample representing 60-day shelf life storage must be tested in duplicate with 15 swatches against S. aureus. The method must be modified to include testing both the fabric and the wash water (5 ml from the automatic washer or 0.5 ml from the container with the test device) in individual widemouth jars containing subculture media and neutralizers. The wash water-to-media volume ratio should not exceed 1:40. Growth or no growth is noted after 48-hr incubation.

(v) No data are required for laundry additives containing sodium-calcium hypochlorites, sodium-potassium dichloro-s-triazinetrione or trichloro-s-triazinetrione as sole ingredients when a concentration of at least 200 ppm available chlorine is provided in the use dilution.

(2) *Textile additives.* Representative treated textiles must be tested as outlined in the current Technical Manual of the American Association of Textile Chemists and Colorists, Method AATCC 30–1971. (See Appendix Part IV.A for sampling protocols.)

(3) *Carpet Sanitizers.* No final official method exists for testing the efficacy of carpet sanitizers; however, a tentative method has been developed by AOAC (Appendix Part IV.A). If this test is employed to derive data to support the registration of a carpet sanitizer, the following protocols must be considered:

(i) Three product samples representing 3 separate batches must be tested against S. aureus and Enterobacter aerogenes with 2 different types of carpet. A 99.9 percent reduction of test bacteria over the control count must be demonstrated in each case. No carpeting is available to serve as a standard. If the product is intended for use on synthetic carpeting, 2 representative carpets, such as acrylic and polypropylene tufted-loop types may be tested. If the product is also intended for use on wool carpeting, a representative sample must be tested. If not, the label must bear a disclaimer for use on wool. All carpet samples tested must be fully identified by the pile fibre type, pile yarn weight of finished carpet, pile density and tuft height. Adequate controls must demonstrate that bacteriostatic agents in the carpet pile or backing do not interfere with the test results.

(ii) One sample, representing a 60-day shelf life stability study, must be tested with one carpet sample and one test bacterium.

(iii) The amount of sanitizer applied to a piece of carpet must be extrapolated to a volume of diluted shampoo per specified area of carpet, and the volume of diluted shampoo must be declared on the proposed label. The volume should be equivalent to 12 gal of dilute product per 800 to 1200 sq ft of carpet. If the application is intended for hospitals, a wet vacuum pickup must be specified in the label directions. In no case will the so-called "dry shampoo treatment" be registered for hospital use. Claims for disinfecting carpets are not acceptable.

(f) *Air sanitizers.* No standard method of evaluating air sanitizers has been adopted. Reports by Stuart and Friedl, McGray, and the British Standards Institution (Appendix Part IV.A) describe methods of testing aerosol products designed for temporary sanitization of air spaces. Several investigators have shown that the incorporation of glycols (triethylene, propylene or dipropylene glycol) at concentrations of 5 to 10 percent in pressurized formulations will significantly reduce airborne bacteria when adequate vapor concentrations are achieved. Currently, then, registration requirements regarding efficacy are satisfied by chemical analysis data showing appropriate concentrations of glycols. Air sanitizing products that do not contain such concentrations of glycols must be evaluated in a room or a chamber; a significant reduction in airborne microorganisms must be demonstrated. The primary test organism is S. aureus. Depending on the intended use pattern, other organisms, such as Salmonella or Pseudomonas, may also be used to provide efficacy data.

(g) *Processing and industrial uses*—(1) *Material preservatives, bacteriostats and self-sanitizers.* Material preservatives, bacteriostats and self-sanitizers defined as pesticides must be carefully assessed as to their practical value relative to the claims made before the products are proposed for registration. Simple demonstration of bacteriostatic activity or reduction of bacterial numbers on bacterial media is not sufficient to establish the practical efficacy of such products. Since there is no recognized standard test for demonstrating efficacy and practical value of material preservatives, bacteriostats and self-sanitizers, it is suggested that written protocols of proposed tests for efficacy be provided for review before the initiation of the tsets.

(2) *In-can paint preservatives.* Chemicals proposed for use as preservatives in paint formulations are identified as pesticides. Paints containing preservatives are not identified as pesticides unless pesticide claims are made or implied. Products proposed for use in preserving water-based paints must show effectiveness in at least 2 representative paint formulations in which the product is intended for use. Tests must be carred out in at least 3 preparations of each of the 2 formulations using pertinent microorganisms and adequate controls. Both test and control samples must be tested for a period of 6 mo to 1 yr. The data must show that no deterioration affecting paint quality occurred during the test period. The American Society for Testing and Materials Test Method D–2574 67T may be referred to for guidance on test protocol. A copy of the test method and a detailed description of the materials and equipment used must be provided.

(3) *Paper coatings, wet-end additives and adhesives.* Data must be provided showing effectiveness in applicable substances with appropriate microorganisms. Tests must be carried out in triplicate with each substance for a period ranging from several days to a year depending on the intended end-use. A complete description of the test protocol must be furnished.

(4) *Plastic formulations.* Data must be furnished to show that the product is capable of inhibiting the growth of bacteria which commonly deteriorate such formulations. For emulsions and liquid formulations, 3 replicate tests with adequate controls must be carred out for a period of at least 5 days. A copy of the test method and a detailed description of the materials and equipment used must be provided. For finished products, zones of inhibition must be demonstrated on seeded agar plates using 5, 1-in-sq test samples of material (providing 20 test edges) for each organism. For formulations which may be highly substantive to the impregnated material, a seeded agar overlay procedure may be required to demonstrate effectiveness. Information on the duration of activity in the finished product must be provided.

(5) *Leather preservation during processing.* Data based on field-derived observations using plate-counts of gross bacterial numbers must be submitted to demonstrate inhibition of bacterial growth and the time interval for which control may be expected.

(6) *Metalworking Cutting Fluids.* The product must be tested in an identified cutting fluid formulation inoculated with Pseudomonas aeruginosa, Enterobacter aerogenes (ATCC 13048), Pseudomonas fluorescens (ATCC 13525), and Staphylococcus aureus, each at a concentration of 1 million cells per ml of cutting fluid. The test must be carried out for at least 60 days with reinoculation after the first 30 days and must demonstrate control or inhibition of bacterial growth at 25–28° C. Three replicate tests must be carried out using adequate controls. A complete description of the test protocols employed must be furnished.

(7) *Antimicrobial fuel additives.* (i) Products proposed for use in this category must show the necessity of the additive for control of bacterial growths in the water phase of the fuel. Generally, kerosene-based fuels subject to water contamination by condensation may be subject to bacterial growths. Jet aviation fuels have been extensively studied with regard to such growths and the attendant deterioration of aircraft fuel tanks. A limited amount of information exists as to the need for control of bacteria in diesel fuels or heating oils stored in metal tanks. No bacterial problems currently exist with respect to aviation or surface vehicle gasolines. With aviation fuel additives, the Federal Aviation Administration (FAA) should be consulted as to the appropriateness of an additive from the standpoint of certification for particular airframes or engines.

(ii) Efficacy data developed using Bushnell-Haas media (Appendix Part IV.A) plus the fuel (the fuel-to-liquid media ratio must be equivalent to that found in the field), inoculated with a mixed culture of bacteria and fungi isolated from the water phase of contaminated fuel and treated at the concentration recommended on the label, would serve to presumptively substantiate the efficacy of a product. A minimum of 3 replicate tests should be carried out for at least 14 days.

(iii) Based on these data, products proposed for use in engines and/or airframes of aircraft would require testing according to the requirements specified in FAA Advisory Circular AC 20–24A, dated April 14, 1967, under an Experimental Use Permit issued by the Registration Division. Where an additive has not been certified for use in a particular aircraft engine and/or airframe, then such use should be disclaimed on the label. It is essential that any collateral technical bulletins which accompany the product be submitted for review.

(iv) Any other proposed uses (diesel fuels, heating oils) would require derivation of efficacy data under an Experimental Permit, after presumptive efficacy was established in Bushnell-Haas media as noted above.

(v) Consideration must also be given to the potential addition of pollutants in exhaust emissions of internal combustion engines using fuels with the proposed additive. The applicant may need to confer with personnel of the Mobile Source Pollution Control Program, Environmental Protection Agency, for additional requirements.

(8) *Antimicrobial additives for sugar mills.* Products bearing claims of effectiveness in controlling bacterial growths in sugar mill operations must be substantiated with both laboratory and field-derived data. Laboratory data showing the effectiveness of the product in inhibiting the growth of or reducing the numbers of representative Leuconostic mesenteroides isolated from spoiled cane or beet sugar pressings must be provided. Field-derived data should be developed under an Experimental Use Permit demonstrating the efficacy of the product used as directed. A food additive tolerance must be established before the product is registered.

(9) *Miscellaneous preservative uses.* Preservatives recommended for use in cosmetics, ceramic glazes, grouts, floor wax emulsions, gaskets (paper felt, cork, rubber, vinyl), films and foams of polyvinyl and polyurethane, dextrin-based inks, photographic solutions, laundry starch, and colloidal graphite should be tested in all end products or representative formulations to substantiate preservative claims. It is suggested that proposed test protocols be submitted for review before initiation of test studies, since no standard methods for evaluating preservatives are available at this time.

§ 162.66 Specific considerations: Aquatic pest control agents.

(a) Applying pesticides to water magnifies actual or potential unreasonable adverse impacts on man and the environment. Contamination can extend to surface waters, ground waters and aquifers used for, or in the production of, drinking, irrigation or industrial process water. Contamination of desirable plants and animals may lead to collapse of a species and/or economic losses. Bioconcentration of pesticide residues in the food chain or direct ingestion of pesticides in water may represent a potential long-term hazard to man. Thus most aquatic uses of pesticides must be covered by an established tolerance or an exemption from the requirements for a tolerance.

(b) *Drinking Water Systems (Human).* The AOAC method for evaluating swimming pool water disinfectants (Appendix Part IV.B) is currently the most applicable standardized test for disinfectants in drinking water.

(c) *Drinking Water Systems (Poultry and Livestock).* Directions for the treatment of drinking water for poultry and other food animals that include or imply claims to control disease in these animals require clearance from the Food and Drug Administration before registration can be issued. Relatively high concentrations of products may be required for effectiveness because of the probability of contamination with feed and fecal matter by the animals themselves and because, in the presence of most forms or organic matter, effectiveness is markedly decreased or eliminated. Claims for the control of specified microparasites and algae may require submission of laboratory and/or field data. Tests must be designed to evaluate the product's performance in contaminated water.

(d) *Weeds.* Performance information on a pesticidal product intended to control aquatic weeds must be derived from in-use tests. The tests must:

(1) Include a geographical distribution of test sites that reflect various water types, temperatures and other environmental factors influencing effectiveness.

(2) Be made with the highest concentration of the product in water which will not injure crops or desirable vegetation if the treated water is to be used for irrigation. This level is to be considered as the highest acceptable for registration.

(3) Establish the concentrations of the product in water (as a result of proposed application) and the decline of that concentration with time and/or distance of flow. It must be shown that the use of treated water can be controlled during the time and/or distance of flow required for the concentration to decline to nontoxic levels. Appropriate statistical analyses of the data and development of depletion curves are essential.

(4) Reports must include the types of aquatic sites, the dosages, the degree of weed infestation, the types of weeds, the physical measurement of the treated area and of the whole site, and the time of each application in terms of date and in terms of the stage of growth of the weeds.

(e) *Fish and mollusks.* (1) Pesticides here may be classified as fish poisons, fish repellents and freshwater molluscicides. These products may be formulated as dusts, granules or sprays. Their effectiveness depends on many variables, including:

(i) The target species.

(ii) The site of application (lakes, ponds, streams, rivers, estuaries).

(iii) The characteristics of the water (salinity, hardness, pH, sedimentary load, temperature, etc.).

(iv) The length of time that the pest organism is exposed to a given concentration of the pesticide.

(v) The concentration or dosages of the pesticide, usually expressed in parts per million and in equivalent amounts of product per unit volume of water.

Their usefulness will be dependent on their adverse effects, such as reducing mollusks as intermediate hosts of parasites of man and other animals.

(2) The following outline summarizes major considerations in testing fish and mollusc control agents.

I. Fish poisons and freshwater molluscicides
A. Laboratory studies
1. Physical-chemical properties of the active ingredient(s)
2. Preliminary screening (static or continuous flow test to show LC–50's at 24, 48, 96 hr or longer using target species. The exact toxicological studies to be performed depend upon the nature of the chemical, method of application, and intended claims. The tests indicated are for toxicants applied to water.)
3. Delineative screening (static or flow test to show LC–100's at 24, 48, 96 hr or longer)
4. Effect of each active ingredient
5. Physiological effect of product
6. Diluent (wetting agents, emulsifiers and synergists) effect on product efficacy
7. Effects of water quality, temperature and turbidity
8. Specificity (species and product action)
9. Phytotoxicity
10. Detoxifying agent
B. Field studies
1. Preliminary field studies (wading pools, ponds, small streams)
a. Biological activity of product at various water qualities for target species throughout their life cycle
b. Method of application and dose effect on target species
c. Method of application and dose levels for partial and/or selective kills
d. Detoxification time (or restocking interval)
e. Phytotoxicity
2. Advanced field studies (Experimental Use Permit may be required.)
a. Regional product testing
b. Application effect and significance
c. Detoxification time (restocking interval)
II. Fish repellents
A. Laboratory studies
1. Physical-chemical properties of active ingredient(s)
2. Acute and subacute toxicity studies on target species (The studies required will depend upon the mode of action of the active ingredient(s), intended claims, method of application and other factors.)
3. Effects of product on individual and group behavior
4. Activity of each active ingredient
5. Dose response to the product
6. Method of application and effect on response
7. Rate, duration and significance of response
B. Field studies

1. Preliminary field studies (ponds, lakes and streams)
   a. Water quality factors affecting efficacy
   b. Other environmental factors affecting efficacy
   c. Method of application and effect on response of target species
   d. Rate and duration of individual and group behavioral change
   e. Phytotoxicity
2. Advanced field studies (Experimental Use Permit may be required.)
   a. Regional testing required
   b. Environmental factors affecting efficacy
   c. Method of application and dose response
   d. Rate, duration and significance of individual and group behavioral change

(f) *Swimming pools.* (1) Disinfectants recommended for use in swimming pool water must be applied at concentrations considerably greater than those recommended for treating human drinking water. Numerous factors influence the concentrations necessary for effectiveness in practical applications: Numbers of swimmers in the pool; frequency of use; frequency with which water is changed; general weather conditions; and types and degree of organic contamination of the water by the swimmers themselves, for example, suntan lotions and oils and various debris.

(2) Swimming pool water disinfectant claims must be substantiated with efficacy data derived from the AOAC Method for Water Disinfectants for Swimming Pools (Appendix Part IV.B). Data from one test on each of 3 different batches against both *E. coli* and *Streptococcus faecalis* are required. Data from duplicate samples of one batch representing a 60-day shelf life stability study against *E. coli* must be submitted. Products marketed as tablets, sticks, briquettes or other slow-dissolving forms require submission of data on solubility rates and field data demonstrating effectiveness in maintaining the required chlorine residual when used as directed.

(3) The preceding data requirements for swimming pool water disinfection do not apply to simple formulations of sodium and calcium hypochlorites, sodium and potassium dichloro-s-triazinetriones, or trichloro-s-triazinetrione when adequate directions for pool water maintenance are provided on the product label.

(4) Algaecide tests are conducted in the laboratory using a recognized standard procedure such as the Fitzgerald test (Appendix Part IV.B). Confirming data are also required from in-use tests in swimming pools of different sizes located in different regions of the country. The number of tests needed will depend on the degree of variability found.

(g) *Industrial Cooling Water Systems.* (1) Products intended to control microbiological slimes in cooling water systems include those with algaecidal, bactericidal and fungicidal activity. Microbiological slimes here means the microorganisms (algae, bacteria and fungi) and their secretions, plus embedded debris. The following terms and definitions are particularly pertinent.

Bactericide. As defined here, the term is restricted to products used in cooling water and must not be related to products represented or defined as disinfectants.

Blowdown (bleed-off). Water discharged from the system to control concentrations of salts or other impurities in the circulating water. Blowdown is measured in gallons per minute.

Closed recirculating systems. Essentially all the water is continuously recycled and the heat is removed from the circulating water in a heat exchanger that may be cooled by air, mechanical refrigeration or a separate open cooling-water system.

Makeup water. Water added to the circulating water system to replace that lost because of blowdown (bleed-off), drift, evaporation, leakage and overflow.

Microbiocide. Any substance or mixture of substances which effectively reduce the number of algae, bacteria and fungi.

Once-through systems. Water is taken from some primary source, used for cooling, and then discharged to waste or used for other purposes.

Open recirculating systems. A major portion of the water used for process cooling is continuously recycled through some type of evaporative cooling device, such as a cooling tower, to remove the heat picked up and is then used again for process cooling.

(2) The data requirements for these products include laboratory data that must be submitted to show the presumptive effectiveness of the product against algae, bacteria and/or fungi. These data may be minimum inhibitory concentration determinations. If the product contains a new chemical or chemicals, the antimicrobial activity, if any, of each such ingredient must be determined against the intended organisms and reported.

(3) Based on the laboratory data, the product must be tested to determine an acceptable dosage range and pattern of use in water systems for which the product is intended. Such testing usually requires an Experimental Use Permit.

(4) Tests should be made in at least 2 different climatic locations for a period of not less than 30 days. Test systems should be representative of the more severe conditions that the product is intended to control. Reports must include, but are not limited to, the following:

(i) Dosage applied at each treatment.

(ii) Interval between treatments.

(iii) Visual or other suitable ratings of the nature and amount of microbial growth. (The rating scale should be defined, and the commercially acceptable level of control should be indicated.)

(iv) Total volume in the system.

(v) Percent blowdown, if applicable, per unit of time.

(vi) The volume of makeup water added to the system during the test period.

(vii) Water characteristics, such as pH, hardness, temperatures, and identification of any anticorrosion inhibitors, together with an estimate of their concentration in the system, and identification of any antimicrobial chemicals that were used immediately before or during the test period.

(viii) All appropriate engineering data reflecting efficient operation of the system, such as heat transfer readings, head pressures and downtime. Baseline data on each parameter used is necessary for comparative purposes.

(ix) Microbial counts before and after treatment to determine the required interval(s) between applications and to indicate the degree of control obtained.

(x) Where, when and by whom the tests were conducted.

(h) *Pulp and Papermill Water Systems.* (1) Treatment of these systems with pesticides may result in residues in finished paper or paperboard. Residues, occurring in finished products intended for use in contact with food, must be covered by a tolerance or an exemption.

(2) Laboratory data must be submitted to show the presumptive effectiveness of the product against bacteria and fungi. These data should include at least minimum inhibitory concentrations and/or count reduction determinations. Standard methods of testing have not been developed; however, tests should be conducted at temperatures frequently encountered in the mill system. Representative species of bacteria and fungi employed in obtaining presumptive effectiveness data must include, but are not limited to, Aspergillus niger (Institute of Paper Chemistry No. 106), Chaetomium globosum (ATCC 6205), Pseudomonas aeruginosa (ATCC 10145) and Klebsiella pneumoniae (ATCC 8308).

(3) Based on the laboratory data, the product must be tested to determine an acceptable dosage range and pattern of use in mill systems. Such testing usually requires an Experimental Use Permit.

(4) Tests should be made in at least 2 domestic papermills manufacturing different types of paper or paperboard, such as newsprint, fine papers, tissue or liner board. Each test should be conducted for not less than 4 wk. Mill conditions should be representative of the more severe conditions usually experienced. Reports must include but are not limited to:

(i) Dosage applied at each treatment.

(ii) Interval between treatments.

(iii) Visual or other suitable ratings of the nature and amount of slime growth. The rating system should be defined and the commercially acceptable level of control indicated.

(iv) Water characteristics, such as pH, hardness, temperature, and presence of nonpesticidal additives (for example, corrosion or foam inhibitors).

(v) Identification of slimicide used before the test, if possible.

(vi) Description of the pulp source (soft or hardwood) and its consistency at the point of application.

(vii) Bacterial and fungal counts before and after treatment, together with visual and tactile observations and operating characteristics (slime breaks, etc.).

(viii) Rate of dissolution of solid form products, such as balls and briquettes,

under the conditions of use in each mill.

(ix) History of slime problems.

(x) Where, when and by whom the tests were conducted.

(5) Similar data developed in foreign countries are acceptable if the data have been obtained in 3 mills where climatological conditions and the microorganisms encountered are like those found in domestic mills.

(l) *Secondary Oil Recovery Systems.* The American Petroleum Institute (API) Recommended Practice for Biological Analyses of Subsurface Injection Water Test (Appendix Part IV.B) is an acceptable method for assessing the efficacy of products against Desulfovibrio desulfuricans (API), Bacillus cereus (API), and Pseudomonas fluorescens (API). The test must be carried out in duplicate, and a copy of the API test procedure must be submitted.

§ 162.67 Specific considerations: Fungicides and nematicides.

(a) The term fungicide as used here means any substance or mixture of substances intended to prevent, destroy, repel or mitigate fungi. Fungus includes any nonchlorophyll-bearing thallophyte of a lower order than mosses and liverworts, such as rust, smut, mildew, mold, yeast and bacteria, except those on or in living man or other animals and those on or in processed foods, beverages, or pharmaceuticals.[2]

(b) The term nematicide means any substance or mixture of substances intended to prevent, destroy, repel or mitigate nematodes inhabiting soil, water, plants or plant parts. It excludes products intended for use against nematodes in or on living man or other animals. Nematode includes invertebrate animals of the phylum Nemathelminthes and class Nematoda, that is, unsegmented roundworms with elongated, fusiform, or saclike bodies covered with cuticle.

(c) Soilborne pests: (1) Tests for fungicides and nematicides applied to soil must be designed to prove effectiveness against different soil-inhabiting organisms present in various soil types under similar and dissimilar climatic conditions. Crop plant safety, or lack of phytotoxicity, is very important and precautions to ensure against harmful effects are often needed in the directions for use. Taste panel tests are frequently important on crops (especially root crops) grown in treated soil.

(2) The control of soil-inhabiting fungi and/or nematode plant parasites may be accomplished with a variety of formulation types and methods of application. Dusts, granules, liquid drenches and fumigants (liquid-to-vapor) may be applied broadcast, in bands over or under the row, in furrows at planting time, or as a transplant or postplant drench treatment; sprays of downward translocating products may also be applied to foliage. All specialized application equipment and techniques should be adequately described.

(3) Data which show beneficial effects such as plant growth or yield increase as the sole proof of effectiveness are unacceptable, and some evidence of pest control and/or symptom repression must also be provided.

(4) Depending upon the fungus or nematode and crop involved, important parameters to be measured may include, but are not limited to, one or more of the following:

(i) Crop emergence.

(ii) Crop and pest mortality or survival.

(iii) Crop stand count.

(iv) Crop lodging.

(v) Crop root injury.

(vi) Disease-free or marketable crop yield.

(vii) Crop yield or growth response.

(viii) Pest and/or symptom expression ratings.

(5) When a general nematode claim is desired, effectiveness data must be submitted to demonstrate control of at least one species of root-knot, cyst, migratory endoparasitic nematode, and ectoparasitic nematode of importance on the crop(s) or crop planting site(s) to be treated If the test results indicate that the product will not control all the important nematodes associated with a given site, claims must be limited to the specific type or types that can be controlled. Nematode control may be effectively evaluated by one or more of the following criteria:

(i) The percent infection or root-knot index—degree of galling on roots of carefully dug plants compared with untreated checks and/or standards (root-knot nematodes).

(ii) Number of nematodes per unit volume or weight of soil compared with untreated samples (all nematodes).

(iii) Number of nematodes per unit measure of roots or other plant parts compared with untreated checks (lesion and other endoparasitic nematodes).

(iv) Number of cysts containing viable eggs and/or larvae in soil and/or on roots compared with untreated checks (cyst nematodes).

(6) The overall results should statistically demonstrate that nematode control achieves a desired quality, quantity or other measurable crop benefit. Pest population densities should be ascertained before treatment, and population comparisons should be made between treated and untreated plots before or at treatment and at proper intervals thereafter.

(7) Proper sampling times will vary depending upon the nature of the chemical and the type of crop and nematode. Final populations in treated plots often exceed those in untreated plots, particularly when early season control results in increased plant growth. For this reason, a trained nematologist is needed for proper evaluation. (See Appendix Part IV.C).

(d) Postharvest fruits and vegetables: The advanced evaluation of postharvest fruit and vegetable treatments requires replicated tests on lots varying in size from several shipping cartons to several tons. Data should be taken after arrival at the destination following actual or simulated packing and transportation procedures for the particular crop to be investigated. When crops are treated before or during storage, the data should give disease counts after varying periods of storage. These counts may be stated in terms of percent of total produce showing symptoms or signs of disease. Usually, separate tests must be conducted on each different fruit or vegetable and for each organism to be controlled. If, however, sufficient levels of 2 or more disease organisms are present on the same host simultaneously, their control may be evaluated in the same test. Disease control data on artificially inoculated produce is also acceptable. The product should be tested with each type of equipment (bin-type dumpers, tank washers, spray washers, brush cleaners, hydrocoolers, and similar devices) which may be named or implied in the directions for use.

(e) Grain preservatives: (1) Fungicides to be used to preserve stored, high-moisture grains can be evaluated in laboratory tests and in-use field-tests. Such testing should include treatments representing a range of dosages and moisture levels. Since high-moisture grain generally implies a moisture content of at least 15 percent, grain moisture may be reconstituted by the addition of water. Moisture levels of 17 to 20 percent will assure more rapid growth of molds and quicker results. Effectiveness evaluations, for both laboratory tests and in-use field-tests, must include comparisons between treated and untreated grain. These evaluations are based on visible mold growth and auxiliary criteria such as temperature rise, respiration rate and weight loss of stored grain.

(2) Laboratory tests should be designed to obtain the presumptive dosage rate(s) necessary to control fungi in or on naturally infested or artificially inoculated grain (mainly with species of Aspergillus, Alternaria and Penicillium). If grain moisture is reconstituted, Fusarium may be used. Experimental units of grain usually range from 1 qt to 25 gal, partially closed glass or plastic containers; 1-qt thermos bottles are often convenient. Tests should be conducted under a variety of controlled conditions.

(3) Once the preliminary efficacy of the product is established in the laboratory, more advanced tests must be conducted under actual in-use storage conditions. Such tests should be performed in either exposed outdoor bins of at least 100-bu capacity, or unexposed outdoor bins (contained within a shed or other similar type of shelter) of at least 10-bu capacity. Tests must contain a minimum of 2 (preferably more) replicates for each treatment. The fungicide should be tested at proposed application rates in several geographic locations where grain is commercially produced and stored. In-use storage testing optimally should begin soon after the grain is normally har-

[2] This statement appears in the amended FIFRA; however, since passage of this law, the classification of bacteria has been changed. Bacteria are no longer classified as fungi, according to Bergey's Manual of Determinative Bacteriology, Eighth Edition, 1974.

vested. The duration of testing should correspond with claims to be made on the labeling (generally 6 to 12 mo). Applicable data should be recorded periodically during storage.

(4) Depending upon the end use of treated grain, additional studies may be required. If use in animal feeds is intended, appropriate feeding studies must be performed, preferably with poultry and cattle, to demonstrate the acceptability of treated feeds. If grain is to be used for planting, laboratory data must be presented to show that treatment does not cause excessive phytotoxicity. Pronounced delays in germination or emergence would necessitate further testing under field conditions. (See also following topic on seed treatments.)

(f) Seed treatments: (1) Seed treatments should be evaluated in either laboratory, greenhouse or field-tests, utilizing heavily infested seed and/or soil known to contain specific, disease-producing fungi.

(2) Soilborne disease control, such as seed decay and seedling blight, may be evaluated in extensive laboratory and greenhouse testing, provided that, where necessary, results are supported by a limited number of field-tests. Extensive geographical distribution of field-tests is not necessary to support claims for seed decay and seedling blight control. In the laboratory and greenhouse testing, disease incidence can be assured by initially slowing germination with reduced temperatures (cold test) or by boosting inoculum levels of specific pathogens. Results are usually presented as percent emergence and percent survival on 2 or more dates while plants are in the seedling stage. Pronounced delays in germination or emergence require field-testing to assure that the treatment does not cause excessive phytotoxicity.

(3) Seedborne disease control, such as blights, rusts and smuts, should be tested under different soil and climatic field conditions in areas where the crop is likely to be grown. Evaluations should be made of emergence, stand, percent disease and yields if applicable.

(4) Treatment should be applied by commercial equipment or reasonable facsimiles which provide the same coverage. In field-tests, well replicated small plots should be used with seeding rates and plant spacings comparable to general cultural practice. The number of replications and the size of each plot should be consistent with the known variability of the soil, the character of the disease and the anticipated disease incidence. Tests in any one location might vary from 4 to 6 replicates of single rod-rows, or equivalent (about 200 plants), for small grains and other small seeded crops, to 4 to 6 replicates of 50 to 100 plants in each of one or 2 rows for cotton, peanuts and other large seeded crops. Larger plots will be needed if samples are to be collected for residue analysis, if grower planting or harvesting equipment is to be used, or if disease incidence is expected to be low.

(5) Tests should be designed to span several planting dates and locations to cover a range of conditions, and usually, to test several of the principal varieties of each crop for phytotoxic response. It is desirable to have seed treatment materials evaluated in regional trials, such as those run by the Regional Cotton Seed Treatment Committee of the Cotton Disease Council.

(g) Ornamental and flowering plants: Final-stage testing of fungicides and nematicides for use on ornamental plants should be conducted in field or greenhouse plots depending on the intended use of the product. Replicated plots of 10 to 1000 or more sq ft each are acceptable. In the case of florists' crops, no detectable injury is usually allowed. Actual counts of disease and nematode incidences are required; the commercial growers' assessment of the product is also valuable.

(h) Bulbs, corms and tubers: Tests to control rots in ornamental bulbs, corms and tubers should be conducted with 5 or more replications of 100 or more bulbs. Apparently healthy bulbs selected from a heavily diseased stock and/or artificially inoculated bulbs should be used for disease control tests; bulbs for phytotoxicity tests should be selected from stocks as disease-free as possible. Disease counts should usually be obtained during and at the conclusion of the storage season. However, testing should usually continue throughout a complete growing and storage cycle to determine the residual activity and phytotoxicity of the product. Effectiveness evaluations are usually based on comparisons between the final yield (weight and/or size and number) of healthy and diseased bulbs within and between treatments. Separate tests should be conducted for each species and each pathogen.

(i) Trees: (1) Requirements for testing products to be used to control tree diseases are highly specialized and must be conducted by experts in that field. These would include public and private qualified forest pathologists and tree-treating experts knowledgeable in:

(i) Exact diagnosis, including symptomological and mycological aspects.

(ii) Method of treatment, including soil and tree injection techniques, sprayers, surgery, and wound dressings.

(iii) Proper evaluation techniques, including statistics where necessary.

(2) Up to 100 or more single-tree replicates of mature specimens of a given species may be required for each treatment and each control group. The number of trees necessary depends on the nature of the disease and the method of treatment. Fewer replicates are required when all specimens are the same age and vigor and growing on similar sites in relation to soil type, available water and drainage, or when transmission of disease is dependent upon climatic factors, such as wind and rain. More trees are required when any of these conditions are variable or when disease transmission is dependent upon an insect vector or the presence of root grafts. Since securing enough trees within a small area is often impossible, the tests must be carefully designed to provide valid results with replicates which may be randomly scattered over one or more contiguous counties within a general geographic and climatic area. In cases where the disease is widely distributed, separate tests from the major geographic and climatic areas will be required, or labeling claims will be limited to the specific areas of test.

(3) For trees grown in nurseries, seed and seedling bed treatments should be conducted using replicated blocks of 9 to 100 sq ft each, depending on the nature and frequency of disease incidence. Again, up to 100 or more single-tree replicates, or replicated blocks of 4 to 20 saplings each, may be required.

(j) Turf: (1) Tests on lawns and other fine turf areas should be conducted on replicated plots of 4 to 1000 or more sq ft. The size of plots and number of replicates depends on disease uniformity, expected severity and the size of application equipment. Sometimes the control of 2 or more diseases can be evaluated in the same test.

(2) To support a general claim for use on turf, lawns or grasses (non-grazing areas), data should demonstrate effectiveness against the same pathogen on at least 4 specimens: 2 cool and 2 warm season varieties of 2 types of grasses (bentgrass, bluegrass, fescue, etc.). Additional tests should be conducted on all other representative grasses likely to be treated to demonstrate that the product is not phytotoxic at dosages equal to and greater than label rates. Products intended to control diseases of Dichondra and other nongrass lawn plants must be tested on the intended hosts under their normal field conditions.

(k) Wood and wood products: (1) Products used to control marine borers, shipworms, teredos, and fungal and bacterial deterioration of raw and finished wood require registration. The products are commonly applied to logs, lumber, plywood, millworked products, paper, paperboard, paper pulp, poles and pilings, timber, wood chips, sawdust, and other wood-based material. Water repellents and sealers which do not claim to protect these products against pests are not considered pesticides and need not be registered.

(2) Numerous wood preservatives can be applied by several methods, each intended to afford protection for various periods of time and extremes of exposure. Effectiveness data must be provided to support each directed method. There are, for example, a variety of industrial applications:

(i) Applications for timber and lumber are usually based on pressure methods of impregnation; occasionally double diffusion techniques are used.

(ii) Sawmill lumber, poles and pilings may be treated by passing the freshly cut pieces through pressure tanks, dip tanks or spray hoods.

(iii) Stored chips and sawdust usually are treated by spraying the material as it moves on a conveyor belt to the storage area.

(iv) Paper pulp is protected by direct addition of the fungicide to the heavy slurry as it enters the storage chest.

(v) Lap stock may be treated by adding the fungicide to the white water system or by spraying the stock on the dry end of the machine.

(vi) When finished paper or paperboard requires protection, the fungicide is usually added as a spray at some point on the dry end of the machine or in a separate operation.

(3) Commercial applications would include dip tank methods to protect sash or other wooden assemblies, or preparations, such as grease and impregnated bandages or injectable liquids, to treat standing poles. As a final example, farm and home applications for treating fence posts and other wood placed in contact with soil would include: 2 or more flowing brush or spray coats; 3-min dips; hot and cold baths; or 12- to 24-hr soaks.

(l) Miscellaneous industrial materials and equipment. (1) Fungicides are used to protect a great variety of products and equipment. The following examples illustrate this diversity; they include broom corn, cellulose sponges, coatings, colors, cordage, drilling muds, dye baths, emulsions, fabrics, fuels, latex, leather, plastics, resins, rubber, and transportation and storage equipment. Directions for use vary with the material or equipment to be protected and with the type of product to be used. Many industrial materials are purchased under a buyer's specification for fungicidal protection, and specific test methods may be required. Products intended for such markets should be tested according to the buyer's specifications and the results should be submitted along with application for registration. Products intended for the open market should also be adequately tested. (See Appendix Part IV.C for suggested methods.)

(2) Information must also be presented showing that when used as directed, under normal conditions, no intolerable adverse effects will ensue. Such effects would include unwanted discoloration, tenderizing of fabrics, other changes in desirable physical characteristics and interference with processes or reactions.

§ 162.68 Specific considerations: Herbicides, plant regulators, desiccants and defoliants.

(a) The term herbicide means any preparation intended for preventing, destroying, repelling, or mitigating unwanted plants or plants identified as weed pests. Herbicides include, but are not limited to, those that work by direct contact, those applied as soil treatments, those applied to plant roots in drainage tiles and sewers, and those applied to algae (algaecide) or any aquatic weeds.

(b) The term plant regulator means any preparation intended for altering the behavior of plants or produce through physiological rather than physical action. Plant regulators include products intended for:

(1) Accelerating or retarding the rate of growth of maturation of plants or plant parts.

(2) Enhancing fruit set, for thinning flowers or fruits, or for preventing fruit drop.

(3) Prolonging or breaking dormancy of plants and plant parts.

(4) Accelerating root formation or the healing of wounds.

Plant regulators exclude nutrient mixtures, soil amendments, or vitamin-hormone horticultural products intended for maintenance and propagation.

(c) The term defoliant includes any preparation intended for inducing or accelerating leaf abscission in plants. The term desiccant includes any preparation intended for accelerating the drying of plant tissues.

(d) Herbicide Terminology: The following specific terminology used in labeling must be supported with adequate data:

(1) Preemergence. The pesticide is applied before emergence of the crop and after planting.

(2) Postemergence. The pesticide is applied after emergence of the crop. The method of application must be described; for example, directed, semidirected or overtop. The interval after emergence may be expressed in terms of time, but the more precise interval in terms of crop stage (and, where appropriate, pest stage) is preferred.

(3) Post-harvest planting. Phytotoxicity data are required to show satisfactory performance of the proposed crops planted in treated soil after normal harvest of original crop.

(4) Preplant. Supporting data and directions for use must specify the interval between application and crop planting. The data must also indicate whether application to unbedded fields will result in an increased concentration of the product accessible to the plant. Adequate directions must be provided on the label to avoid excessive concentrations.

(e) Rotational cropping and emergency replantings: (1) Crop plant species commonly planted in rotation on treated areas must be identified and the acceptable interval between treatment of the previous crop and the planting of each rotational crop must be given.

(2) Frequently, climatic conditions prevalent after herbicide treatment hinder crop development, and reseeding may be necessary. In this event, recommendations concerning replanting must specify the time and conditions under which the original crop, or an alternate one, may be planted, and whether an additional herbicide treatment is required.

(f) Special noncropland data requirements: (1) The term noncropland is a general category designating areas which are not normally used to grow food, feed or fiber crops. Noncropland sites vary greatly in the degree of contact by humans, domestic animals and wildlife and in the type of, or absence of, desired vegetation. On the basis of these differences, intended noncropland management practices may be classified according to the extent of weed control; that is, highly selective, selective, or nonselective weed control.

(i) Highly selective weed control would be practiced on sites with relatively high concentrations of humans and no restrictions on contact by domestic animals or wildlife. Sites include ornamental turf and shrubs around the home and recreation areas, golf courses, nurseries, highway plantings, areas around farm buildings, agricultural fence rows, urban utility distribution lines and easements.

(ii) Selective weed control would be practiced on sites which are not regularly frequented by relatively high concentrations of humans and which have no restrictions on contact by domestic animals and wildlife. Sites include forest areas, shelter belts, highway and utility rights-of-way and rain runoff drainage ditches.

(iii) Nonselective weed control would be practiced on sites in which contact by humans, domestic animals and wildlife is restricted by physical barriers. Sites include certain industrial areas, lumber yards, tank farms, and areas around utility substations and inside security fences.

(2) Performance data requirements for these sites may be categorized as follows.

(i) When the product intended for use on the first type of site is already registered for use on crops for which tolerances are established, and the dosages, patterns of use and limitations are identical, no further performance data are required.

(ii) When highly selective weed control is not intended, data requirements for products include:

(A) performance data to show the effective dosage range for control of the pests claimed and the dose-response relationship, which may be demonstrated most easily in the form of a probit or regression analysis.

(B) mobility and soil persistence data, correlated with phytotoxic response evaluation, which can be presented by regression curves.

(g) Herbicide data development. Data requirements are discussed under General Considerations. Herbicide tests should be conducted to determine the dosage range within which the product is effective without producing undesirable injury to the crop or nontarget species on adjoining sites. Control plots are included for treatment comparisons. The types of plots depend on the objectives of the experiment and may include cultivated checks, weedy checks, and/or standard treatments. Appropriate statistical analysis is usually essential for adequate interpretation.

(h) Plant regulators: Tobacco sucker control: Plant regulators useful for control of suckers on tobacco require special methods for their testing and evaluation. For protocols, see Appendix Part IV.D.

§ 162.69 Specific Considerations: Invertebrate Control Agents.

(a) Invertebrate pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any invertebrate pest present in the environment. Invertebrates here comprise any of the

numerous small arthropods belonging to the class insecta; other allied arthropods, including spiders, mites, ticks, centipedes, millipedes and wood lice; and terrestrial invertebrates, including slugs and snails. (For aquatic invertebrates, see Specific Considerations: Aquatic Pest Control Agents; for nematodes, see Specific Considerations: Fungicides and Nematicides.)

(b) In addition to organic and inorganic chemicals, invertebrate pesticides include such substances as biological control agents, chemosterilants, hormones, pheromones and attractants. Test procedures and experimental data relating to the efficacy of these substances are presently considered on an individual product-by-product basis.

(c) Special comments for testing insecticides follow. One precaution to observe in any insecticide treatment is the potential harmful effect on honey bees and other beneficial insects during field-testing. Tests should be conducted to determine deleterious effects, and any observation on injury to any beneficial nontarget organism should be reported in the data.

(d) Foliar plants: (1) When testing nonsystemic insecticides, new plant growth, which must be protected against insect attack, and new populations of insects, which have hatched or moved onto the plant since the last treatment, dictate repeated applications for the length of time and at intervals that protection is needed. Uniform coverage also is essential to good insect control. Thus tests must be designed to determine correct dosages and application intervals.

(2) Testing for use on food crops in home gardens must follow the same techniques outlined under General Considerations for the product's commercial use. (See also Appendix Part IV.E.)

(e) Dormant plants: Treatments to dormant plants should be applied so that the insecticide will contact the overwintering stage of the pest or a residue of the insecticide will be deposited where it will be effective against the emerging pest.

(f) Livestock, poultry, fur and wool-bearing animals: (1) Insecticides which are tested for use on livestock, poultry, fur or wool-bearing animals should not injure the animal even when application is repeated frequently over a long period of time; the margin of safety to the treated animal is a vital consideration in determining usefulness. Neither should the insecticides appear illegally in or on meat, meat byproducts or eggs. If the treated animal is less than 3 mo of age, stress conditions created by a particular method of treatment must be evaluated along with the toxicity data.

(2) Certain petroleum oil fractions are used extensively as components in formulations to be used on livestock. In testing such formulations, the possible effects on the animal from toxicity of the petroleum oil must be considered. For example, small amounts of some solvents such as xylene and toluene may cause tolerable minor itching and burning for short periods after application. Higher concentrations, say 25 percent, may cause death if not diluted before application. Based on past experience, certain criteria for testing these compounds have been developed:

(i) Ready-to-use oil solutions of insecticides recommended for use on livestock as mist sprays generally must be applied at rates not exceeding 2 oz per large animal (cattle, horses, etc.) and 1 oz per animal for small animals (sheep, swine, goats, etc.) on a per-day or per-application basis.

(ii) Pour-on and other ready-to-use products containing oils must be tested to ensure that the recommended method of treatment will not result in excessive dosages of oil which may evoke adverse reactions in treated animals.

(iii) Emulsifiable concentrates must be tested to ensure that the final use dilution does not contain dangerous amounts of oil.

(3) Dust bag products and devices should be tested in protected, dry areas, since the dust will often tend to harden when wet and render the product ineffective. A suitable discussion of the formulations, unit locations, and characteristics of the device, such as amount dispensed, type of construction, length, and porosity, should be included in the testing procedures for these applications.

(4) Dusts may also be applied manually to livestock as neck and back treatment or by dusting the entire animal. Some test chemicals may be applied lightly on the surface of the hide; others must be rubbed in thoroughly to penetrate the hide and reach the skin.

(5) Pests of dairy cattle include various biting flies (horn and stable flies), lice, ticks, houseflies, face flies, mosquitoes and sarcoptic mange mites. Evaluating an insecticide that is to be applied directly to the cattle to control these pests should be based on adequate small-scale tests on representative animals from dairy herds in at least 5 widely separate regions where the insecticide is to be marketed. Tests should follow a prescribed schedule using dosages both above and below the anticipated optimum. The probable levels of dosage and concentrations needed should be identified in laboratory or small-scale tests before the field-testing. Although it is usually necessary to repeat the application only once or twice for lice, ticks and sarcoptic mange mites, a schedule of repeated applications is normally required against flies and their continuous population pressures.

(6) Because untreated control herds are usually impractical to maintain, the effectiveness of the test product may be measured by comparative treatments with registered products applied to portions of the test herds. Pretreatment pest counts are an essential aspect of the test protocol. Records should be submitted on the comparative changes in milk production; and insect counts following each treatment to evaluate the effect of treatments.

(g) *Lawns and ornamental turf:* Lawns and ornamental turf are treated with insecticides by broadcast applications. Dusts may be applied with a hand duster or lawn fertilizer spreader; granules and dry baits, with a lawn fertilizer spreader; wettable powders and emulsifiable concentrates (after dilution with water), with a compressed air or knapsack sprayer, a hose proportioner or a watering can. It is important to observe any phytotoxic effects which might occur after treatment, since grasses or other lawn plants differ significantly in their sensitivity to chemicals, and some high quality lawn and golf greens represent a sizeable monetary investment.

(h) Forest lands and roadsides: The use of insecticides on forest lands and roadsides (parks and picnic areas) is frequently under the supervision of Federal or State forest services or county or municipal public officials. Whenever possible, testing insecticides for use in these areas should be in close cooperation or consultation with these officials.

(i) Mosquito abatement: (1) Mosquitoes are among the most important arthropods that transmit disease and annoy man. Numerous species are involved, and the number is compounded by marked differences in their breeding habits, behavior, and dispersal. Consequently, control measures for mosquitoes, whether larvicides or adulticides, usually must relate to several species or to a species group to be applicable to mosquitoes in general. Experimental checks should consist of untreated areas.

(2) Insect resistance can be a major problem when testing a product for mosquito control. Other factors of special concern in determining a product's usefulness are wildlife and fish hazards, possible water or air pollution, effects on house paints and spotting of automobiles.

(3) Repellency from mosquito coils, candles and torches is dependent upon the concentrations and duration of smoke or fumes which permeate a limited area. The test results on products of this type should demonstrate repellency of mosquitoes and other small flying insects on patios, porches or other confined outdoor areas where there is little or no breeze. Testing for human and animal disease prevention or control is possible only in well coordinated, large-scale area programs, such as those conducted by public health agencies.

(j) Human and pet treatments. Insecticides of this type may be applied as sprays, dusts, lotions, towels, ointments, creams, sticks, flea collars, flea tags, soaps and shampoos. Insect repellency tests should demonstrate minimal contact between the insect and the test chemical. Reports must distinguish between killing and repelling effects, since repellent insecticides may preclude any lethal effects. Because breed, age, size and hair length influence the usefulness of insecticides applied to dogs and cats, all these factors should be evaluated in testing pet treatments.

(k) Premise treatments.

(1) *Household insecticides.* In general the presently adopted and tentative official test methods of the Chemical Specialties Manufacturers Association (CSMA) are followed in testing house-

hold insecticides. These methods are The Peet-Grady Method; The Aerosol and Pressurized Space Spray Insecticide Test Method for Flying Insects; Cockroach Spray Method; Cockroach Aerosol Test Method; Textile Resistance Test; and The Determination of Plant Safety of Pressurized Formulation for Use on Plants in the House or Garden. (See Appendix Part IV. E.)

Test data must be submitted to verify that there is no staining of other adverse effects to wallpaper, paint, tile, varnish or other finished surfaces likely to be contacted by the product, when applied according to label directions for use.

(2) *Commercial and industrial insecticides.* These formulations may be in liquid or pressurized form for spray treatments and in paste, powdered or granular form for bait treatments. So-called crack and crevice spray applications should effectively control the target pest and destroy isolated populations without exceeding a maximum coverage of 20 percent of the floor and lower wall surfaces, including shelving and cabinets. Commercial and industrial insecticides are generally tested in the same manner as household insecticides, but it is important to be extremely cautious or, if possible, avoid their use in or around food or food processing areas.

(3) *Food handling establishments.* (i) A food handling establishment is any place, other than a private residence, in which food is held, processed, prepared and/or served. Food areas include those used for receiving, serving, storing (dry, cold, frozen, raw), packaging (canning, bottling, wrapping, boxing) and preparing (cleaning, slicing, cooking, grinding) food; for edible waste storage; and for enclosed processing systems (mills, dairies, edible oil extractors and evaporators). Non-food areas include garbage rooms, lavatories, floor drains (to sewers), entries and vestibules, offices, locker rooms, machine rooms, boilers roms, garages, mop closets and storage (after packaging).

(ii) Any registration for uses in food handling establishments will require associated food additive tolerance regulations as stipulated in Section 409 of the Federal Food, Drug, and Cosmetic Act.

(4) *Agricultural insecticides.* (i) Insecticides to be tested in or around dairy barns, horse barns, stables, poultry houses and yards, and other agricultural structures may be applied as sprays, dusts or baits to stanchions, walls, floors, manure, bedding, and other areas where the target insects may rest or breed.

(ii) To control flies in agricultural structures, residual sprays should be applied to wet all resting places to the point of runoff. Outside areas where flies gather, such as corrals and paddocks, should be sprayed thoroughly.

(iii) If insecticides are to be tested as space sprays, fogs or vapors in dairy barns, horse barns, shelter sheds, milk sheds, or other agricultural structures, animals should be removed and air circulation should be reduced as much as possible by closing doors, windows and other openings.

(iv) Insecticide baits may be tested as cakes, cones, syrups and liquid or dry formulations in agricultural structures and in outdoor areas. Liquid and dry baits should be applied as a certain weight or volume per specified area of floor or ground. Cake or cone products should be placed or hung in areas frequented by the target pests.

(v) Products intended to be used in poultry houses and yards are generally tested as sprays or baits on manure, window sills, outside surfaces of penned enclosures and on the floor of feed and storage rooms. They must be applied in a manner that precludes illegal residues in feed or drinking water.

(l) Structures. (1) Insecticide treatments include wood preservatives and termite soil poisons. Both subterranean and dry-wood termites cause major property damage particularly across southern areas of the United States. Tests on the effectiveness of wood preservatives and soil poisons as termite treatments must be carried out over a minimum of 6 yr under actual use conditions to be meaningful and acceptable; no reliable accelerated testing procedures exist at this time. Soil treatments require that special care be exercised to assure that water supplies are not contaminated with the chemical and that the treatment causes no intolerable injury to foundation plantings of shrubs and other ornamental plants.

(2) Tests for use of wood preservatives to control powder post beetles, old house borers, carpenter ants and certain other wood-destroying insects may follow conventional test method procedures.

(3) Testing by professional individuals or agencies can sometimes be arranged to gather data in the latter stages of development of many of these products.

(m) *Stored products.* (1) Commodities to be protected include grain, cereals and other dry foods, tobacco, woolen and feather goods, furs and furniture stored in such places as grain elevators, boxcars, ship's holds and warehouses. The evaluation of insecticides here is usually based on a high percentage reduction in the various insects after treatment and on assessment of any possible adverse effects on the grade or quality of the treated commodity. In certain cases—with furs, woolen and feather goods—complete protection is essential. In all cases where food or feed commodities are involved, tests must be conducted on palatability. If seeds are involved, data on germination or viability must be provided.

(2) Space sprays and residual treatments should be tested in such a manner or in locations which minimize the contamination of stored feed, food or tobacco.

(3) Fumigants release toxic vapors and should be tested only after man, beneficial animals or birds have been evacuated from the premises. Fumigated premises should be thoroughly ventilated before reentry.

(n) *Fabrics.* (1) Tests of products to be used against fabric pests, such as clothes moths and carpet beetles, must demonstrate complete control of the insects.

(2) Temporary moth treatments are nonresidual sprays which kill by contact action and which require repeated applications. Semipermanent mothproofers are residual chemicals applied to wool, fur, or hair rugs, garments and blankets as coarse sprays or, in some cases, in drycleaning solutions. To test semipermanent mothproofing sprays, the articles should be sprayed lightly but uniformly until the surfaces are moist, but never soaked or saturated. Dry the sprayed articles thoroughly before testing.

(3) Permanent mothproofers are impregnated in the fabric during the dye process and should remain effective throughout the life of the fabric. Permanent formulations should be tested before and after repeated washing or drycleaning. (For mothproofing test procedures, see Appendix Part IV.E.)

§ 162.70 Specific considerations: Vertebrate Control Agents.

(a) Vertebrate pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any vertebrate animal which the Administrator declares to be a pest. Vertebrate include all wild and domestic mammals (except man), birds, amphibians and reptiles. (For fish, see Specific Considerations: Aquatic Pest Control Agents.)

(b) Vertebrate pesticides include lethal agents; irritants; repellants based on odor, taste, postingestional psychophysiological reaction or pharmacological reaction; repellants based on mechanical action such as tackiness or stringiness; anesthetizing chemicals, reproductive inhibitors; and fumigants.

(c) The outline at the end of this section summarizes the basic information needed to evaluate efficacy of vertebrate pesticides. Additional data may be required where conditions, such as pattern and location of use, claims made or implied, method of application, species and subspecies differences, indicate the data are insufficient to answer specific questions. For the total data requirements for any substance or mixture of substances submitted for registration as a pesticide, review Chemistry and Hazard Evaluation. Some interpretative comment on test data and test design parameters follows.

(d) Test data. (1) The albino laboratory rat and mouse are recognized as invaluable in screening and studying vertebrate pesticides; nevertheless, acceptable evidence of efficacy of new compounds and new uses must, in the final stages of the study, include the exact animal species for which control claims are made in the labeling. There are several examples wherein members of closely related groups exhibit markedly different susceptibility to the same compound. Alpha-naphthyl-thiourea (ANTU), for example, has a LD–50 of 8 mg/kg for the brown rat and a LD–50 of 220 mg/kg for the roof rat. The differences in susceptibility need not be of this magnitude, however, to affect label claims. The well-known anticoagulants, as an example here, may require 10 or more

days feeding to control a brown rat (Rattus norvegicus) population, but the same formulated bait may require 15 or more days to achieve comparable control with house mice (Mus musculus).

(2) In most cases, acceptable data on the species claimed must also cover both sexes and both young and old animals, to determine the influence of age and sex on susceptibility to the proposed product.

(3) Although data on lethal dosage are very important, species' physiological differences, food preferences, and feeding habits may play an even more important role in a product's efficacy. Bird feeding habits, for example, are largely governed by sight and tactile perception. Even small changes in customary size, shape, texture or color of food can be enough to reduce the initial rate of bait acceptance.

(4) Results of tests, in which a chemical is administered forcibly (by intubation) or by *ad libitum* feeding, and no alternative untreated foods are available, are useful as pharmacological data and as guides in formulating finished baits. Such tests should be supplemented by free-choice bait acceptance studies since wild vertebrate populations nearly always have a free food choice. Treated and untreated portions of the same food may be used to determine acceptable levels of toxicant in the bait. Tests to determine the acceptability of a formulated bait should be compared to a standard placebo diet, where such a standard has been established. Statements regarding the palatability of ready-prepared baits must be supported with appropriate bait acceptance studies using the target species.

(5) When vertebrate pesticides are to be applied to plants or seeds, data must be submitted showing that the proposed formulation does not produce phytotoxicity or significantly reduce seed viability. Such studies should be conducted on representative plant or seed species and should reflect the effects of application on plant growth or seed development through a growing cycle. The data must also reflect effects of repeat applications where such applications are recommended.

(6) If vertebrate pesticides are to be applied to food or feed crops, the establishment of finite tolerances, or exemptions from the requirements for tolerances, may be necessary.

(e) Test design: (1) Study teams should include personnel who are thoroughly familiar with the life habits and behavior of the target species. This is particularly important when the goal is repellency and the criterion for evaluation is a change in an established behavior pattern. For example, if an attempt is made to rid an airport of gulls with a chemical repellent used just before they would habitually migrate to northern nesting grounds, the result is questionable.

(2) When designing efficacy tests for use under cage or captive conditions, corroborative methods of field application should be kept in mind. For example, if an area repellent is intended to keep dogs off a lawn or a garden by treating the perimeter, then merely demonstrating that a dog will avoid a familiar object when treated with the product is not applicable proof of area repellency. Similarly, a so-called repellent that appears to have value in stopping the use of a urine scent-post may have little value in stopping some other objectionable behavior.

(3) Research and development of vertebrate pesticides should always include tests conducted under actual field conditions. The interrelationship between inter- and intraspecies behavioral patterns and the impact of environmental factors are so complex that cage tests alone cannot suffice.

(4) If a claim is made that a product is effective for a particular time period, then the test design must reflect studies covering that period of time. For example, several bird repellents that rely on the continued tack-and-string of a bead of gel that has been applied to a roost, claim effectiveness for one year. The registrant is responsible for tests to verify that the gel does not crust or otherwise lose its effectiveness for a full year under different climatic and physical conditions. Similarly, claims that an indoor pet repellent does not stain upholstery or that a rodent population can be satisfactorily controlled in 10 or more days must be supported by appropriate test data.

(5) The outline is grouped by type of study (laboratory studies, preliminary field studies, and advanced field studies). The laboratory studies should provide the preliminary data to indicate efficacy of a potential product or use. Preliminary field studies should indicate the product's potential in terms of effect on small areas or populations, dosage ranges under field conditions, and individual and group animal reactions to a treatment. Advanced field studies should show, on a regional basis, the efficacy of the product under a variety of use conditions, environmental factors, etc. (The number of regional studies required vary, depending upon the species claimed and their normal range, the claims made in the labeling, etc. Therefore, no statement can be made to cover all situations which will be encountered.)

I. Laboratory studies (preliminary efficacy studies)
A. Physical-chemical properties of active ingredient(s)
B. Acute and subacute toxicity studies using target species (The exact toxicoligical studies required will depend upon the mode of action of the chemical(s), the method of application and the claims.)
C. Effect of each active ingredient
D. Physiological or behavioral effect of product
E. Dose response to the product
F. Rate and duration of response and/or physiological effects
G. Cage test—single and multiple animal studies using target species (Cage studies for repellents will vary with the intended action of the chemical and the behavior to be modified. Because of the many variables involved in testing repellents, it is advisable to submit the proposed test protocol for review before implementation.)
1. Food preference studies
2. Product acceptance and reacceptance
3. Effect of product age on acceptance
4. Effects of stickers, spreaders, diluents, etc.
5. Specificity to target species and/or sex
6. Effects of age on susceptibility
7. Activity cycle effect on animal susceptibility
8. Special claims
9. Effect of method of application on response (where applicable)
10. Phytotoxicity (where applicable)
H. Product stability (shelf life)
II. Field studies
A. Preliminary field studies
1. Test and control areas required
2. Pre- and post-treatment population census and census technique
3. Rate, duration and significance of response
4. Effect of environmental factors (climatic and physical) and managerial practices
B. Advanced field studies (Experimental Permit may be required.)
1. Regional testing required
2. Pre- and post-treatment population census and census technique
3. Rate, duration and significance of response
4. Effects of environmental factors (climatic and physical) and managerial practices

Label Development

§ 162.71 General comments.

(a) The information discussed as general comments is basic to all pesticide products. The specific comments on types of products (for example, aquatic pest control agents, fungicides, etc.) provide only additional detailed information related to a particular type of product. Labeling requirements are specified in § 162.10.

(b) Claims. (1) Claims for the use of a pesticide product relate to the pest or pests to be controlled and, sometimes, to the mode of action of the product. Claims are derived from the data obtained in performance testing and modified, as necessary, by the usefulness data, the information developed on the product's chemistry and its hazard evaluation. The modification process results in (i) directions for use; (ii) limitations of use; and (iii) appropriate caution statements. Each claim must be clearly defined, must reflect the data and must not extend beyond the demonstrated value of the product. Such claims as eliminate, eradicate, exterminate, free and rid, and any claim that the product is safe are too broad and promising and are unacceptable.

(2) Claims Subject to Regulation Under FIFRA, as Amended: Whether a product is intended for use as packaged or after dilution or mixture with other substances, such as water, kerosene, talc, clay, fertilizers, baits, or other carriers, solvents, diluents, or propellants, it will be considered a pesticide if:

(i) Claims or recommendations for use as a pesticide are made on the label or labeling of the product.

(ii) Claims or recommendations for use as a pesticide are made in collateral advertising, such as publications or advertising literature which does not accompany the product, or radio or TV advertisements.

(iii) Claims or recommendations for use as a pesticide are made verbally or in writing by representatives of the manufacturer, shipper, or distributor of the product.

(iv) The product is intended for use as a pesticide after reformulation or repackaging.

(v) The product is intended for use both as a pesticide and for other purposes. (Such a product is subject to all provisions of the Act, including section 2(q)(1)(A) under which a product is misbranded if its labeling bears any statement that is false or misleading concerning any pesticidal or nonpesticidal use.)

(3) Claims subject to regulation under other acts: (i) Whenever application is made to EPA for registration of a product that is both a drug and pesticide, and the determination is made that the principal claims or representations relate to pesticides, EPA will withold registration of the product until the Food and Drug Administration confirms that the product complies with the provisions of the laws administered by that agency.

(ii) Whenever application is made to EPA for the registration of a product that is both a drug and a pesticide, and the determination is made that the principal claims or representations relate to a drug, EPA will refer the application to FDA for processing.

(iii) Neither agency will approve the marketing of a product under the law administered by that agency if the product does not comply fully with the requirements of the law administered by the other agency.

(iv) Although the claim for use in federally inspected meat, rabbit, poultry and egg processing plants will be accepted on labeling, registrants must obtain clearance for such use of their products from:

U.S. Department of Agriculture, Meat and Poultry Inspection Program, Animal and Plant Health Inspection Service, Washington, D.C. 20250.

or

U.S. Department of Agriculture, Egg Products Inspection Programs, Agricultural Marketing Service, Washington, D.C. 20250.

(4) Claims for manufacturing use only: (i) This phrase is permitted on the product label provided it is qualified by wording on the front panel which identifies the type of manufacturing use (that is, formulation of other pesticides, repackaging use, or incorporation into other manufactured products as preservatives or protectants against pests); and type of pesticide involved (fungicide, herbicide, insecticide, disinfectant, etc.). For example:

(A) For manufacturing use only in formulating fungicides and bactericides.

(B) For manufacturing use—for repackaging as insecticides.

(C) For manufacturing use—as a bacterial and fungal preservative for coatings and adhesives.

(ii) The guidelines enumerated below are intended to clarify registration requirements.

(A) Pesticides intended for formulation of other pesticidal products.

(1) Labels for products in this category should bear the identifying phrase, For Formulation of (Insecticides, Fungicides, Disinfectants, etc. * * *), on the front panel. This phrase would normally apply to an unformulated pesticide intended to be processed into another pesticide product by, for example, encapsulation, incorporation into or onto granules, addition of adjuvants, or by simple dilution.

(2) The label or a technical bulletin to which the label refers must provide adequate information concerning the physical and chemical properties of the chemical that are pertinent to the needs of formulators. This requirement may be waived when, as in the case of a common, well-known pesticide, such information is generally available.

(3) Proof of safety and utility as a pesticide may be waived in the case of well-known pesticides. In other cases, the registrant of formulating-use-only products has 2 options:

(i) Submit proof of effectiveness as to the type of pesticide(s) named, based only on presumptive laboratory data. In this case the registrant must include a statement on the labeling notifying formulators that they will be responsible for providing supporting data for their formulations; or

(ii) Submit XYZ or John Doe labels for various formulations together with confirmative data on the results of safety and efficacy tests for each product formulation. The statement in item 1 above notifying formulators is not required.

(B) Pesticides intended for repackaging. These products would not require further processing or formulation. The front panel of the label should bear the phrase, For Repackaging of a (Fungicide, Herbicide, Rodenticide, etc. * * *), and refer to a technical bulletin. This bulletin should include: Specimen labeling giving adequate directions for use based on a registered label of identical formulation; reference to laboratory and in-use efficacy and toxicity data which have been submitted in support of the above registered label; reference to the requirement that any new uses or any further reformulation of the product may require additional supportive laboratory and/or efficacy and safety data; and reference to the requirement that data may be necessary if any modification of a pesticide product, such as simple dilution, addition of dyes or perfumes, nozzle designs, or any other change that could affect the hazard or performance of the product, is involved. In lieu of the technical bulletin, the preceding information must appear on the label itself.

(C) Pesticides intended for use in items that are not pesticides. Products in this category should bear the label claim, For Use as a (Fungicide, Bactericide, Bacteriostat, etc. * * *) in (Coatings, Fabrics, Building Materials, etc. * * *). The products should be recognized preservatives for items or materials that are not themselves identified as pesticides. A technical bulletin should provide: Information on pertinent physical and chemical properties of the pesticide product, such as leaching, photodegradation, volatility and stability in treated items; and directions for use, including a general description of the intended purpose of treatment, method(s) of application, dosage and relative safety to the environment. Detailed directions for use may be omitted from the label provided that: Such a technical bulletin is provided; the pesticide will not come into the hands of the general public except after incorporation into finished products; and in the opinion of the Administrator, such directions are not necessary to protect the general public.

(D) Pesticides intended for use in 2 or more categories. These products must satisfy the labeling requirements of each intended category, stated separately on the label and in the technical bulletin or bulletins.

(E) Pesticides intended for use to control pests in, on, or around industrial sites are excluded from any of the categories listed above and must be registered with adequate directions for use given on the label or in a brochure accompanying the product.

(5) Claims as Preservatives: (i) The terms preservation, preservative, preserves, when used alone, are interpreted to be pesticide claims, and the products are subject to registration under the Act. The general fields of usefulness must be named and specific organisms may be identified; for example:

(A) Preserves wood against fungal rots and decay.

(B) For the preservation of oil-in-water emulsions. Effective against Aspergillus niger, Pseudomonas fluorescens, P. aeruginosa and Staphylococcus aureus.

(C) Preservative for leather. Effective against a wide range of bacteria and fungi.

(ii) If pesticide use is not intended, the words preservation, preservative of preserves must be modified by specifying the nonpesticidal action intended; for example:

(A) Preserves the color (shape, texture, etc.) of * * *

(B) When used as a preservative on metallic surfaces, protects against corrosion.

(iii) The terms preserves, preservation, and preservative used in conjunction with shoe polish, saddle soaps and leather conditioners to indicate benefits derived from water repellents and sealers, such as epoxy, silicone and wax formulations which do not contain active antibacterial or antifungal agents, are not considered pesticide claims.

(c) Directions for use: (1) The label directions should specify what is to be controlled, where or on what it is to be controlled, and how the product is to be applied, at what rate, when and with what frequency. For example: The names of the pests or pest groups and directions for their control should be associated with the appropriate crops or sites of application; the label should indicate if a product is for use in greenhouses and include greenhouse directions

and dosage rates; and the label should indicate if a product is intended for aircraft application and contain appropriate directions for use, including the minimum amount of water or other diluent to be applied per acre.

(2) Directions should be appropriate for the size of the package on which they appear. The directions for home use should not require more of the product than is contained in the package.

(d) Limitations of use: Use limitations should be closely associated with the appropriate directions for use and crops to which they refer. Limitations on the uses of pesticides relate to directions for use and the persistence, toxicity or physical characteristics of the individual product. Certain directions for use, such as apply by aircraft or apply to aquatic sites (lakes, ponds, streams, swamps) will impose a classification restriction and limit the application of that product to certified applicators. The persistence and toxicity of a product are reflected in such limitations as: (1) The maximum dosage and time of last application before harvest of a feed or food crop consistent with an established tolerance; (2) an established regulation on field reentry by workers having intimate contact with the treated crop; and (3) the safety and legality of planting subsequent crops on treated land. Limitations imposed by the physical characteristics of a product include, as an example, restrictions on the use of butyl esters of phenoxy herbicides because of their volatility.

(e) Precautionary statements: Precautionary statements warn the public of hazards which may be encountered in handling, using, storing or disposing of a product. Hazard may be dependent on toxic or irritant properties, on availability of antidotes, or on explosive, flammability or other special characteristics. Appropriate first-aid treatments and antidotes, if available, should appear in the precautionary statements. See Packaging, Storage and Disposal for additional information.

(f) Labeling requirements for pre-measured unit packaging:

(1) Pre-measured unit packages of pesticides so marketed will be required to exhibit the following labeling on the individual unit package:

(i) Statement of active ingredient(s).

(ii) The appropriate signal word(s) as well as the skull and crossbones, if appropriate.

(iii) The EPA Registration Number.

This information must appear in 6 point type or larger. The manufacturer may, at his option, add additional information to the package when size permits, providing it does not detract from the prominence of the required information. This requirement applies to the smallest individual unit of a pesticide packaged separately which could be expected to be removed from the outer container. If a water-soluble package is enclosed in a foil or plastic package on a one-to-one basis, only one of the 2 packages will require the above labeling as either one would be the smallest individual unit which could be expected to be removed from the outer container.

(2) The outer container label for all pre-measured unit packages must bear the following statement in a prominent position: "Do not remove packages from container except for immediate use".

§ 162.72 Specific comments on antimicrobial agents.

(a) The brand name and the type of product identified on the label must be consistent with the claims made and the use directions provided. The claims made for a product must be related to the level of effectiveness (sterilizer, sporicide, disinfectant, sanitizer, bacteriostat), the microorganisms affected and the pattern of use.

(b) Self-sterilizing, self-disinfecting, actively germicidal, and self-sanitizing claims for surfaces and treated materials are frequently proposed. Claims of this type have led to a great deal of confusion and deserve special attention. The resistance of bacteria to chemicals in the absence of moisture virtually rules out the possibility of producing self-sterilizing, self-disinfecting or actively germicidal materials or surfaces. Bacteriostatic and self-sanitizing materials and surfaces may be produced as the result of treatments with chemicals; however, the practical value of such treatments must be considered as a mitigating level of activity and must not be classified as protective in preventing the spread of infectious microorganisms. The presence of moisture is required for growth of microorganisms as well as for the germicidal activity of chemicals. For this reason, chemicals that occur as a dried residue after application of a disinfectant to an inanimate surface or that exist in impregnated materials exhibit antibacterial activity in the presence of moisture but would have only minimal, if any, activity in the dry state. Obviously, such a claim as permanently germicidal for a treated surface could not be justified. In general, claims made for residual bacteriostatic and self-sanitizing activity of treated surfaces or materials are being discouraged because of the unrealistic test procedures currently available and the lack of practical usefulness and public health significance of the product in actual use.

(c) The claims germproof or germproofed, referring to any surfaces, materials or articles, indicate the existence of actively germicidal or self-disinfecting properties. Since there are no known pesticides that have been shown to provide these benefits, these claims are not permitted on the label or labeling of disinfectant products.

(d) The unqualified claims kills germs and kills bacteria may be considered by the consumer as synonymous with sterilization. Since no differentiation is made with respect to the type of germs which will be killed, the purchaser has a right to expect that the product will kill all germs and all bacteria, including the most resistant bacterial endospores. These claims are badly misused in advertising media. The claims kills most germs, kills many germs, kills most bacteria and kills many bacteria may be appropriate for many disinfectants, provided that laboratory or test data developed with both Gram-positive and Gram-negative organisms support such claims.

(e) Claims for usefulness against specific diseases and specific infectious bacteria, fungi and viruses are commonly proposed. It is considered misleading to claim that applications of any germicide or disinfectant to inanimate surfaces will be effective against any specific disease. A disease is a condition, and the words, typhoid, tuberculosis, cholera, poliomyelitis, anthrax, etc., describe specific pathological conditions. When used in labeling and advertising, they imply that the product may have value in the treatment of the specific condition or conditions named, but this is seldom, if ever, true. Claims for effectiveness against the specific causative agents of diseases, however, are acceptable, if true.

(f) In the registration of products, such superlative claims as ideal and perfect are objectionable. The word ideal denotes perfection, and it would be unfair to allow a manufacturer to portray his product at this standard of excellence. It should be deleted, or replaced with a designation such as good, especially adapted or excellent, whichever may be true.

(g) Names coined from the word sterilize, such as Steri, Steryl, etc., are considered to imply that the product is a sterilizing agent and are objectionable.

(h) Phenol Coefficients: A phenol coefficient claim constitutes a disinfectant claim. An unqualified phenol coefficient claim is interpreted to represent a value against Salmonella typhosa. Where a phenol coefficient is declared against any other bacterium, the phenol coefficient against S. typhosa also must be declared. Phenol coefficient claims are not acceptable for acid toilet bowl cleaners, halogens, alkalis, alcohols, formaldehyde and potassium permanganate.

(i) Virucides: The claim virucidal is not acceptable unless qualified according to the specific viruses or types of viruses against which the product has been tested and shown to be effective. A virucidal claim must also include the phrase on environmental surfaces.

(j) Effectiveness in hard water: (1) If claims for effectiveness in hard water are made, the label must indicate the hard water tolerances claimed: for example, Effective in waters up to _____ ppm hardness calculated as calcium carbonate; or, to meet the requirements of the U.S. Public Health Service Ordinance and Code, This product fulfills the criteria of Appendix F of the Grade A Pasteurized Milk Ordinance, 1965 Recommendations of the U.S. Public Health Service in waters up to _____ ppm of hardness calculated as calcium carbonate when tested by the AOAC Germicidal and Detergent Sanitizers—Official Method. When it is shown that the hard water values materially differ, as between S. aureus and E. coli, the lowest value must be stated. In this case, the respective

hard water values may also be stated separately.

(2) Any product that bears label claims for effectiveness in hard water must be tested by the appropriate method in hard water at the tolerances claimed.

(k) Air sanitizers: (1) Glycols and glycol mixtures sold to temporarily reduce the number of microorganisms in the air are subject to the provisions of the FIFRA, as amended. With glycols and glycol mixtures sold for the use in continuous vaporizing devices, the vaporizer itself need not be registered; however, since its use is stipulated as an integral part of the directions for use of a specific pesticide, the labeling used in connection with the sale of the vaporizer is collateral to the labeling of the pesticide and must be submitted as a part of the registration. Label directions must stipulate that all doors and windows be closed, the length of time the product should be released into the air and the appropriate percentage relative humidity necessary for the product to be effective.

(2) Adequate experimental data is available to show that, irrespective of the method of dissemination, air sanitizers will not sterilize, disinfect, act as a germicide, or protect experimental animals from infection by airborne bacteria and viruses. They will be accepted for registration when the claims are made in such a manner as to clearly indicate the mitigating nature of the activity of glycol vapors continuously dispensed and when adequate directions for the maintenance of effective atmospheric conditions and vapor concentrations are furnished.

(3) With dispensers for the intermittent treatment of air, such as aerosols, no appreciable amount of scientific data exists in the literature to indicate that products of this type have any practical value, except under specific situations, because of the rapid rate of disappearance of glycol vapor from the air.

(4) Claims like the following are unacceptable for air sanitizers: Kills Cold and Flu Germs in Midair; Destroys Microbes that Spread Measles, Mumps, Pneumonia, Strep Throat, Colds, Flu; Protects Homes, Offices Against Infectious Diseases; Gives Protection to Office Workers; Effective Cold Treatment; Germ Killing; Air Disinfection; Prevents the Spread of Acute Respiratory Infections; Sterilizes Air; Purifies Air; Germicidal Vapors; Bactericidal Vapors.

(5) The phrases Fresh Air, Refresh the Air, Air Freshener, Provides Fresh Air are objectionable in that use of these products cannot be considered as an efficient substitute for adequate ventilation in closed spaces, and such products do not provide fresh air. Qualified terms such as Provides Refreshing Fragrance would be acceptable. The term fresh when used with the word air requires a prefix and a suffix (refreshner) to avoid connotations of providing fresh air.

(6) The label of products concurrently represented as surface disinfectants and air sanitizers must clearly delineate and separate the claims and directions for surface disinfectants from those of air sanitizers.

(7) Fogging applications of disinfectants cannot be accepted to disinfect air or room surfaces. Thus, such label representations as germicidal fogging, decontamination and disinfectant fogging are not acceptable. Recommendations for fogging applications of chemicals to sanitize surfaces may be accepted when directions specify that this use is an adjunct to accepted methods of manual cleaning and disinfecting.

(l) Antimicrobial Fumigrants. (1) Either disinfection or sterilization may be accomplished under certain conditions by the use of gases or vapors. The chemicals most commonly used are formaldehyde and ethylene oxide. Complete directions for use must be provided to stipulate the amount of gas or vapor necessary, the cubic feet of closed space to be treated and the exposure time required to accomplish the intended end result.

(2) Sterilizing processes using ethylene oxide require a pretreatment to rehydrate dry spores which may be present.

(3) If pretreatment, relative humidity, temperature or other factors are essential to the effective use of any of these chemicals, requirements must be clearly specified on the label.

(m) Laundry additives. (1) A clear distinction should be made between products recommended for household laundering and products represented as commercial laundry additives. Directions for household laundering products may require different dosages for front-loading or top-loading automatics and/or wringer-type washers. Product dosages in this instance must be stipulated as household measures. The fabric-to-water ratio in home laundering is about 1:10, whereas in commercial laundering the ratio is 1:5. The effectiveness of certain products is significantly altered because of these differences; thus, demonstrated efficacy in one system cannot be extrapolated to the other.

(2) Dosage directions for use in commercial laundering may be based on pounds of dry cloth. To determine the aqueous concentration of the product, each pound of dry cloth is converted to 5 lb of water. The use of sours or acid salts or fluorine and/or acetic acid with acetates in the final rinse requires determination of efficacy with the sour present in test solutions.

(3) Label claims must be keyed to the actual levels of activity that have been tested and substantiated, and where bacteriostatic and/or sanitizing/disinfecting claims are concurrently made, the label text and directions must clearly delineate and separate the respective levels of activity. Claims for residual self-sanitizing activity on treated fabrics must be related to the presence of moisture; for example, imparts a self-sanitizing residual on treated fabric, active in the presence of moisture would be acceptable.

(n) Toilet bowl and urinal disinfectants and sanitizers: (1) Claims for disinfecting the hidden trap and claims for solutions in the flush tank to disinfect the bowl each time the toilet is flushed are unacceptable. Drip feeder systems, liquid chemicals and deodorizing blocks are not accepted as disinfectants or sanitizers for such fixtures, although there is no objection to deodorizing claims for such chemicals. Where it is demonstrated that a practical level of bacteriostatic odor-controlling activity can be maintained in the water reservoir or traps, such claims may be acceptable.

(2) If directions for use of the product specify, Force the bowl water over the trap, liquid concentrates recommended for direct application to the bowl can be accepted for disinfecting, *Provided*, That additional directions specify swabbing all surfaces thoroughly. The product may be tested, undiluted, against *Salmonella chloraesuis* by the AOAC Use-Dilution Method.

(3) When residual water is present in the bowl (approximately 96 fl. oz), a concentration high enough to kill S. choleraesuis in the AOAC Use-Dilution Method must be specified with additional directions for swabbing all bowl surfaces.

(4) The directions for use of toilet bowl sanitizers must also specify swabbing all surfaces thoroughly and may be tested in a manner similar to disinfectants by the appropriate surface sanitizer test.

(o) Chemical toilets: Lye, coal tar emulsions, cresylic acid-soap, phenolic-soap, phenolic salts, orthodichlorobenzene and quaternary ammonium compounds have been registered for use in chemical toilets. No claims for disinfection of the excreta itself are acceptable. The products may, however, be identified as disinfectants. If such identification is made, the directions for use must include instructions which will adequately disinfect the container. Claims related to human wastes must be restricted as, for example, to aid in the sanitary disposal of human waste.

(p) Dust mop and cloth treatment compounds: Claims of effectiveness must be restricted to bacteriostatic action on the treated mop or cloth, and the label text must clearly distinguish between the bacteriostatic activity and dust retention. Directions for use must be sufficient to yield a finished mop or cloth that can be shown to the bacteriostatic by test and efficacious as a vehicle for gathering and holding dust particles.

(q) Mattresses and upholstered furniture: Fumigation or gaseous sterilization is the only effective and practical means to sanitize, disinfect or sterilize pillows, mattresses, upholstered furniture or similar dense objects. Disinfectants are not effective for these uses and claims for such products are unacceptable. Some components of furniture, however, such as fabrics, leather, plastics, burlap and ticking, can be effectively treated to resist microbial deterioration before fabrication into furniture. For additional information, see the discussion on material preservation.

(r) Bird cage and animal litters: In controlling odors in animal rooms, in bird houses in zoological parks, in bird cages and in pet litter boxes in the home, it has become common practice to treat the litter with chemicals to prevent the growth of odor-producing bacteria. Products with claims for odor control only can

be registered on the basis of valid, simulated in-use data submitted by manufacturers.

(s) Household and institutional (nonmedical): An unqualified representation that a product is intended for use in institutions is too broad in that it may include or imply use in medical as well as nonmedical institutions. The requirements to substantiate efficacy will be applied on the basis of medical use where the unqualified word institutional appears on the labeling.

(t) Hospital and related institutions: (1) A hospital disinfectant must be tested against *Pseudomonas aeruginosa*. If it is not effective against this test organism, the product still may be accepted for registration bearing hospital, medical, dental, clinical and veterinary representations. However, in this case, the label must bear a prominent disclaimer to the effect that the product is not effective against *Pseudomonas* species. The label disclaimer may be replaced by special directions needed to assure efficacy against this organism if certain concentrations or applications in a special manner are required.

(2) No activity short of disinfection is considered to have practical significance in critical medical areas, such as infectious wards, isolation areas, operating rooms and intensive care units. Products not identified as disinfectants and recommended for use in hospitals must be restricted to use in noncritical hospital areas and must bear a disclaimer such as, This product is not a terminal disinfectant and must not be relied upon as a disinfectant for critical hospital areas.

(3) When products are sold or recommended for use in the disinfection of surgical instruments, directions for use must include instructions for cleaning all instruments to remove adhering blood, pus and serous exudates before immersion in the solution recommended. This is critical for hinged instruments or other devices containing small orifices, tubes or seams.

(4) Oral and inhalation equipment used in hospitals and related institutions are reported, with increasing frequency, to be vehicles for disease transmission. The moist environment in components of the equipment tends to maintain the viability of microorganisms that are deposited there following use by the patient. If a product is recommended for disinfection of such equipment, it should be effective against *P. aeruginosa* and *Mycobacterium tuberculosis* as well as *S. aureus;* efficacy against bacterial spores may be required in the future. Because of the serious nature of this problem, whenever practicable, such equipment should be disassembled, precleaned and sterilized, and label directions should include this recommendation.

(5) Claims for disinfection of human wastes are unacceptable, although disinfectants may be recommended to aid in the sanitary disposal of such wastes and to disinfect the containers themselves after cleaning.

(6) Deodorants used in the hospital environment are generally products that simply mask the odors associated with patients suffering from incontinence, draining wounds, severe burns, etc., and are not practical in controlling or inhibiting microbial growth. However, some bacterio-static deodorants have been accepted for hospital use where claims of value are clearly defined. Care must be exercised to avoid confusion of deodorizing activity with disinfection.

(u) Barber shops and beauty parlors: (1) Adequate cleaning and disinfection of instruments and equipment between customers should be recommended on the label. Products manufactured for these uses include bacteriostats, sanitizers, and disinfectants.

(2) Use of such products is usually controlled by local ordinance. The requirements are far from being uniform, and much of the wording employed is inconsistent with any reasonable law enforcement program. Sterilizing requirements are almost always confused with sanitizing and disinfecting processes. Thus, it is impossible to draw up a registration policy that will meet all of these health requirements and not conflict with certain specifications.

(3) The basic policy employed is that a disinfectant for this use should be effective against both enteric and pyogenic bacteria. If fungicidal claims are made in the labeling, the product must be effective against pertinent pathogenic fungi as well as bacteria.

(4) Difficulties have been enecountered with anhydrous lubricants for clippers. No disinfecting claims have been accepted for such products, although representations as *bacteriostatic cleaning oils* have been accepted.

(v) Funeral homes, mortuaries and morgues: Various products, such as deodorizers, sanitizers and disinfectants representing a rather wide variety of special chemicals, are used in these establishments. Products that are represented solely for embalming bodies and that make no pesticidal claims are exempted from the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. However, products sold for use on instruments, equipment, furnishings, preparation and autopsy rooms and associated vehicles are subject to the Act and must be registered. Exorbitant claims for protection of personnel are unacceptable for these products.

(w) Restaurants, Taverns, etc.: (1) Various disinfectants, germicides, sanitizers and detergent-sanitizers are recommended for use on cooking utensils, dishes and glasses in restaurants, taverns, bars, lunch counters and other establishments where food or beverages are served. Some of the products serve as germicidal rinses for previously cleaned dairy and food processing equipment as well as for dishes and glasses in restaurants.

(2) The basic requirements for such products, except for those used on interstate carriers, are stipulated in various local Public Health Ordinances which vary widely as to detail. The U.S. Public Health Service has provided a Model Ordinance and Code which makes certain recommendations in this field. Many local ordinances, however, differ in their specifications from this Code. Thus, the general policy has been to develop labeling requirements based on modified claims, so that all products for which there is specific public health demand in some locality can be legally transported in interstate commerce.

(3) Emphasis is directed toward the differentiation between sanitizing and disinfecting reflected in the definitions employed. Although the most desirable type of directions for sanitizing or disinfecting should specify washing, rinsing in water and rinsing in the chemical solution, directions specifying two-stage processes are accepted; that is, washing and only rinsing in the chemical solution.

(x) Farm uses. (1) Products which are recommended for use on farm premises are divided into 2 major categories, food and nonfood, as determined by the intended use and the claims which appear on the label. The guidelines for registering farm-use items which fall into the nonfood-use category are the same as those applicable to general household and institutional (nonmedical) uses.

(2) Whenever the recommended uses of a product are such that residues of the chemical are likely to contaminate animals which may be a source of food, feeds for such animals, or food products derived from animals, the product is identified as a food-use item. These products must be cleared through the Food and Drug Administration. If the applicant cannot submit evidence that his product has been cleared for the food uses shown on the label, the product will not be registered under the proposed label. The applicant may desire to revise his proposed label so that the product can be identified as a nonfood-use item. This is generally accomplished by specifically directing the user to take appropriate steps to avoid any contact of the product with food animals, feed and water, feeding and watering devices and containers which might be used for the byproducts of animals as a source of food. Removing animals from premises before disinfection of surfaces and thorough rinsing, after treatment, of watering and feeding devices is usually adequate to place the product in the nonfood-use category. For example, the following precautions are required for nonfood-use farm premise disinfectants containing cresylic acid or synthetic phenols:

(i) Do not use in milking stalls, milking parlors or milk houses.

(ii) Remove all animals and feeds from premises, cars, boats, trucks and other equipment.

(iii) Remove all litter and manure from floors, walls and surfaces of barns, pens, stalls, chutes and other facilities and fixtures occupied or traversed by animals.

(iv) Empty all troughs, racks and other feeding and watering appliances.

(v) Saturate all surfaces with accepted disinfecting solution.

(vi) Immerse all halters, ropes and other types of equipment used in handling and restraining animals as well as forks, shovels and scrapers.

(vii) Ventilate buildings, cars, boats and other closed spaces. Do not house livestock or employ equipment until treatment has been absorbed, set or dried.

(viii) Thoroughly scrub all treated feed racks, mangers, troughs, automatic feeders, fountains and waterers with detergents and rinse with potable water before reuse.

(3) The following special label directions are required for poultry house disinfectants to permit their classification as nonfood-use products. These directions must appear on labeling of products containing cresylic acid, synthetic phenols, pine oils or quaternary ammonium compounds.

(i) Remove all poultry and feeds from premises, trucks, coops and crates.

(ii) Remove all litter and droppings from floors, walls and surfaces of facilities occupied or traversed by poultry.

(iii) Empty all troughs, racks and other feeding and watering appliances.

(iv) Saturate surfaces with recommended solution.

(v) Ventilate buildings, coops and other closed spaces. Do not house poultry or employ equipment until treatment has been absorbed, set or dried.

(vi) Thoroughly scrub treated feed racks, mangers, troughs, automatic feeders, fountains and waterers with detergents and rinse with potable water before reuse.

(4) The Food, Drug, and Cosmetic Act authorizes the setting of tolerances for residues of pesticides which may legally remain in or on food or feed. The procedure for establishing tolerances through the Registration Division of the Environmental Protection Agency is given in detail in Petitions for Tolerances.

(5) Flank and udder washes and teat dips: (i) Products which are recommended solely for use as antibacterial agents on the living body of man or animals are not subject to the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. They may, however, be subject to regulation by the Food and Drug Administration.

(ii) Many pesticides which are recommended for use on inanimate surfaces on dairy farms bear additional label directions for use as teat dips and flank and udder washes for dairy cattle and farrowing sows. The acceptance for registration of labeling of any pesticide recommended for use on living animals as an antibacterial treatment will be contingent upon clearances for these uses by the Food and Drug Administration.

(6) Egg washing and sanitizing. Products recommended for cleaning and sanitizing eggs are divided into 2 categories: Those used on eggs for human consumption and those used on hatching eggs only. Applications on eggs for human consumption are identified as food additives and clearance must be obtained from the Registration Division, EPA. Products recommended for use on hatching eggs are not identified as food additives. Claims of effectiveness from egg washing and/or egg dips generally are restricted to the benefit (reduction of bacteria) derived during the treatment period. Any additional claims for residual control activity and/or improved hatchability compared to untreated eggs must be supported with reliable test data. (See also Antimicrobial Agents, Sanitizing Rinses.)

(y) Processing and industrial uses: (1) *Dairy, Food and Beverage Plants.* (i) The basic requirements for products in this category are based on the Grade "A" Pasteurized Milk Ordinance recommended code of the U.S. Public Health Service as set forth in Public Health Service Publication No. 229. This publication is available from the Government Printing Office. Products proposed as sanitizers for aplication in or on surfaces or articles coming in contact with food in dairies, bakeries, breweries, canneries, meat packing houses, slaughter houses, poultry processing plants or any other food-processing industry require essentially the same efficacy tests and label directions for use.

(ii) In dairy sanitizer applications, local ordinances differ in their specifications from the USPHS Model Ordinance and Code. Thus, modified claims and recommendations specific for a particular locality may be acceptable on proposed labels.

(iii) In general, products in this category require a clear differentiation in the label text between disinfecting and sanitizing. The directions must stipulate thorough precleaning with soap or detergent, rinsing, followed by immersion, flooding or spraying with the terminal sanitizing treatment. Where disinfecting claims are made, separate directions must be provided that stipulate modes of application and the product concentration that would assure disinfection. In registering iodophors, label references to concentrations of iodine must be expressed as titratable iodine.

(iv) Sanitizers applied to food contact surfaces are defined as incidental food additives under the Federal Food, Drug, and Cosmetic Act and require establishment of a food additive tolerance. A list of chemical agents for which such tolerances have been established may be obtained from:

Commissioner for Compliance
Food and Drug Administration
Department of Health, Education, and Welfare
Washington, D.C. 20201

(v) Products recommended for use in meat, poultry and rabbit processing plants may also require authorization for use in federally inspected establishments. Applications should be sent to the following address:

U.S. Department of Agriculture
Meat and Poultry Inspection Program
Animal and Plant Health Inspection Service
Washington, D.C. 20250

(vi) Applications for products recommended for use in egg-processing plants should be sent to:

U.S. Department of Agriculture
Egg Products Inspection Programs
Agricultural Marketing Service
Washington, D.C. 20250

(vii) Acceptance of a product for use in officially inspected plants does not convey with it a license to claim or imply approval or endorsement by any Federal agency. A label representation, such as authorized by USDA for use in _____, with the naming of the types of establishment(s) or plant(s) for which the product has been authorized, can be accepted.

(2) *Preservatives for raw foods or feeds.* Products intended for use on or in processed foods or beverages are not subject to FIFRA, as amended, and do not require registration. Products proposed for use on raw agricultural commodities as preservatives must confer a mitigating or growth inhibiting level of antimicrobial activity when applied directly to such foods or feeds. No claims for sanitizing or disinfecting can be considered. The main intent of use is to control mold growth; however, in certain instances control of bacteria may also be intended. The applicant should carefully consider the primary intent of use and make label claims accordingly. If the chemical agent proposed is a known food preservative, such as sorbic acid, potassium sorbate, propionic acid, sodium propionate, sodium benzoate or an acceptable antibiotic, no test data to substantiate claims are required. Where the proposed product contains a new chemical, test data must be submitted in representative seeded food or feed substrates to show inhibitory growth activity against pertinent microorganisms. The product must have a food additive tolerance or an exemption from a tolerance from the Environmental Protection Agency. In all cases, minimal directions for use must be furnished on the product label or in collateral labeling.

(3) *Material preservatives, bacteriostats and self-sanitizers.* This category includes products bearing claims of value in preserving an article or material from bacterial degradation. The chemical agent used in the article or material is defined as a pesticide, but the treated material is not. However, treated articles or materials that bear or imply antimicrobial claims of effectiveness outside of the material itself are pesticides subject to all provisions of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. These distinctions are not well understood and as a consequence unwarranted claims of effectiveness for many products have been made. Chemical preservatives impregnated in certain materials, for example, have been found to leach out of the treated materials and lose their practical usefulness.

(4) *Paper coatings, wet-end additives and adhesives.* The label for preservatives intended for use in adhesives, wet-end additives, glues, sizing, etc., must be specific in describing the substance to be preserved; for example, starch-based adhesive, protein-derived adhesive, animal glue, corn starch solution, etc. The effective dose range for each type of sub-

stance to be preserved must be specified in the directions.

(5) Textile additives. The label directions for such products must clearly differentiate between preservative action and antimicrobial activity outside the treated material itself. Adequate directions must be provided either on the label or in collateral labeling that describe how the product should be added to the textile material, the limiting chemical or physical factors that should be noted in the application, and the type(s) of textile material for which the product is intended.

(6) Antimicrobial additives for sugar mills. Since cane or beet sugar processing is continuous, label directions must include proportioning pump delivery rates of product, per ton of sugar produced, per 24 hr.

§ 162.73 Specific comments on aquatic pest control agents.

(a) *Drinking water systems (human).* (1) Water-treating chemicals should not be designed as sterilizers, and only claims for water sanitizing and/or water disinfection can be justified. Presently chlorine-type products are the disinfectants of choice in treating human drinking water and drinking water systems. The directions for treating the water itself, however, and the directions for treating the various parts of the water system should be differentiated. Iodine-type products may also be used for field or emergency treatment of drinking water.

(2) The amount of available chlorine necessary to disinfect drinking water will depend directly upon the chlorine demand of the water supply. Thus, it is not practical to attempt to be precise with respect to the minimum concentration which must be recommended. In general, recommendations should provide for a free residual available chlorine concentration of at least 0.2 ppm throughout the system. The amount of chlorine which must be added to accomplish this concentration will vary widely and may be as high at 10 ppm. With residuals higher than 2 to 2.5 ppm, taste becomes a factor in acceptance. Chemical control tests for free residual chlorine are necessary and must be specified on the label.

(3) With iodine, the concentration required to disinfect water will vary from 0.6 to 4.3 ppm, depending upon such factors as temperature and pH. For emergency treatments, 8 ppm should be sufficient to take care of water with iodine demands up to 4 ppm.

(4) For disinfecting standpipes and water mains before use, 50 ppm of available chlorine with a holding period of 24 hr should be specified.

(b) *Drinking water systems (poultry and livestock).* Care must be exercised to differentiate between claims of value in disinfecting the water and claims of value as a medication for the animal. Medication claims identify the product as a drug as well as a pesticide and must be cleared with the Bureau of Veterinary Medicine of the Food and Drug Administration.

(c) *Weeds.* Control of weeds in irrigation systems, lakes, ponds, rivers and streams means the reduction of unwanted weeds or algae to a level that will not interfere with optimum water management and navigation. The directions for use must include:

(1) The sites intended to be treated.

(2) The types of weeds (floating, emersed or submersed) intended to be controlled.

(3) The common names of weeds to be controlled, such as cattails or water hyacinth. For algae, only the type need be stated (blue-green algae or filamentous green algae).

(4) The dosage appropriately expressed. For application to moving bodies of water, use weight or volume of product per unit volume of flow per unit of time.

(5) The method of application, such as broadcast (for granules), surface spray or subsurface injection. Surface spray application also requires that the name of the diluent, if any, the concentration, and any necessary limitations on spray droplet size (for example, 400- to 800-micron) be specified.

(6) The time of first application in terms of stage of growth of the weeds or season of the year.

(7) The number of applications per year and the interval between treatments.

(8) Any necessary limitations on the use of the product or of the treated water, such as:

(i) The area or fraction of the site to be treated at any one time to avert fish kill.

(ii) The interval between last application and the use of the treated water for drinking by man and domestic animals, or for irrigation, crop spraying, fishing and recreation.

(d) *Fish and mollusks.* (1) Fish control product labels must name the individual species to be controlled, unless the performance data clearly demonstrate effectiveness against an entire group of fishes. Trash fish will be unacceptable unless the data shows effectiveness against representatives from all groups.

(2) Marine molluscicide product labels must specify the organisms (for example, oyster drills) to be controlled. Labels for freshwater molluscicide products, conversely, may make a general claim for freshwater snails. (See also topic on antifouling paints.)

(e) *Mosquito Larvicides.* (1) Because these products are applied to lakes, ponds, estuaries and swamps, involving relatively large areas, special precautions are usually required on the labeling, particularly in respect to hazards to fish and wildlife.

(2) Label claims for human and animal disease prevention or control are not acceptable on household or other products for limited application; for example, around bird baths, fish pounds or farm ponds.

(f) *Swimming pools.* (1) Available chlorine, from both inorganic and organic compounds, is the most commonly registered disinfectant for swimming pool water. Specific directions for the chlorination of unstabilized swimming pool water with inorganic compounds must provide for a minimal available chlorine residual of 0.6 to 1 ppm to be maintained at all times. In addition, the directions should provide for a superchlorination procedure for newly filled pools. Instructions to test pool water with test kits, to maintain the proper available chlorine level and to maintain a pH of 7.2 to 7.6, must also appear on the label. Representations that a product will maintain chlorine residuals for lengthy periods of time without frequent checks with a chlorine test kit are unacceptable. It must be emphasized to the consumer that the maintenance of a chlorine residual is dependent on ambient temperatures, light intensity and bather load.

(2) Most organic and some inorganic chlorine-bearing chemicals are recommended for use in the presence of a chlorine stabilizer. There is ample evidence to show that the concentration of residual chlorine needed to maintain effectiveness must be increased in the presence of various concentrations of stabilizing agents. Thus, those chlorine products recommended for use with stabilizing agents must specify a minimum maintenance dose range of 1 to 1.5 ppm residual chlorine in lieu of data from controlled field trials sustaining the validity of lower concentrations. Claims of effectiveness at a sanitizing level and claims of effectiveness against athlete's foot fungi in footbaths are unacceptable for such products.

(3) Iodine and bromine products are employed less frequently as swimming pool water disinfectants. Similar pertinent directions for use are required for the registration of these products.

(4) No evidence has been developed to show that quaternary ammonium compounds will provide the minimum level of effectiveness required by public health officials, and these products are unacceptable as swimming pool water disinfectants. They have been accepted, however, for sanitizing and disinfecting pool associated surfaces when the requirements as surface-treating chemicals have been met. Quaternary ammonium compounds also have been registered as algae control agents in swimming pool water.

(5) To register swimming pool algaecides, the directions for use must specify:

(i) The place of application; for example, apply to the water along pool edges.

(ii) The types of algae intended to be controlled.

(iii) The dosage and pattern of use. This will usually include, but is not limited to:

(A) Initial dosage (or dosage range) when pool is free of visible algae.

(B) Initial dosage (or dosage range) when the pool contains visible algae growth. The pattern of use must specify reapplication of initial dose at stated intervals until algae growth is visibly controlled and the debris is removed.

(C) The maintenance dosage (or dosage range) and the intervals between maintenance dosages.

(g) *Industrial cooling water systems.* (1) Control of bacteria, fungi or algae within cooling water systems means to stabilize the microbial population at such a level that slimes will not interfere with system efficiency.

(2) Broad claims for use, such as microbiocide, microbiostat, slimicides and microorganism control, are acceptable only when they are modified by (i) naming the types of organisms (algae, bacteria, fungi) for which control is intended, and (ii) identifying the industrial or commercial recirculating water cooling systems; for example, industrial (and/or commercial) recirculating cooling water towers, air washers and/or evaporative condensers. Such terms as biocide or biostat are unacceptable where only microorganism control is to be construed.

(3) Cidal claims are acceptable only where tests demonstrate that the product effectively reduces the number of living algae, bacteria or fungi. Static claims are to be made for products where tests demonstrate that they provide temporary control or growth suppression. The claim for a type of activity (cidal or static) must be related to each particular use claimed on the labeling. Examples include: microbiocide for use in industrial recirculating cooling water towers to reduce the number of living algae, bacteria and fungi; and microbiostat (microorganism control) for use in industrial recirculating cooling water towers to control the growth of algae, bacteria and fungi.

(4) Directions for general microbial control must include, but are not limited to, the following:

(i) Where in the system the product is to be applied; for example, at a point in the system where it will be uniformly mixed.

(ii) The timing of application; for example, when the system is in jeopardy of becoming impaired or after cleaning a system whose efficiency is already impaired.

(iii) The dosage to be applied. The dosage rate for solid products must be specified in weight of product per unit volume of water. Labels of products formed into tablets, briquettes or other uniformly sized shapes should state the weight of each unit. If the dosage rate for liquid products is stated as fluid volume, or parts per million, of product per unit volume of water, the product weight per gallon must be shown on the application. If, however, liquid products are added on a weight basis, the product weight per gallon must be shown on the label.

(iv) The method of application or pattern of use. An initial application dosage, which may be repeated, is followed by subsequent lower dosage applications. The initial dosage range is to control noticeable contamination, to satisfy the demand of the system which may have extensive but not noticeable contamination or to rapidly develop a desired level of toxicant in the system water. The subsequent dosage range is to inhibit the growth of microorganisms remaining or subsequently introduced into the system. In case of extensive reinfestation of the system, the initial dosage range should be applied until control is again evident. Examples of various methods of application or patterns of use are given below.

(A) *Intermittent or slug method.* The directions must stipulate an initial dosage range of relatively high product concentrations to obtain control of obvious microbial contamination. When microbial control is evident, a subsequent dosage range, of relatively lower product concentrations, must be stated. The label must state the usual time interval between doses, or include the phrase, as needed to maintain control, or both. The label must not claim maintenance of any particular pesticide concentration. Example of direction for use:

Initial Dose. When the system is noticeably fouled, apply ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water in the system. Repeat until control is achieved. Badly fouled systems must be cleaned before initial treatment.

Subsequent Dose. When microbial control is evident, add ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water in the system every ------ day [weekly], or as needed to maintain control.

(B) *Modified intermittent method.* This is similar to the intermittent or slug method except that the interval between treatments is based on the time for a stated fraction of the system water to be lost by blowdown. This method of application may allow a claim to maintain the pesticide concentration within stated maximal and minimal limits. Example of direction for use:

Initial Dose. When the system is just noticeably fouled, apply ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water in the system. Apply half [or ⅓, ¼, or ⅕] of this initial dose when half [or ⅓, ¼ or ⅕] of the water in the system has been lost by blowdown. Badly fouled systems must be cleaned before initial treatment.

Subsequent dose. When control of microbial growth is evident, apply ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water in the system. Apply half [or ⅓, ¼ or ⅕] of the subsequent dose when half [or ⅓, ¼, or ⅕] of the water in the system has been lost by blowdown.

(C) *Continuous feed method.* The label should specify an initial slug dose, based on the volume of water in the cooling system, to establish an initial product concentration. For volatile products, continuous feed is based on total makeup water. For nonvolatile products, continuous feed is expressed as amount of product to be applied per stated volume of water lost by blowdown. The claim to maintain a pesticide concentration in the system may be made for this method of application. Example of direction for use:

Initial Dose. When the system is just noticeably fouled, apply ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water in the system. Badly fouled systems must be cleaned before initial treatment.

Subsequent Dose. Maintain this treatment level by starting a continuous feed of ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ------ [unit volume] of water lost by blowdown.

(D) *Briquettes or Tablets.* These may be considered slug dose methods of application. Data must be submitted showing the rates of dissolution of the product in water at various temperatures and flow rates. Example of direction for use:

Slug dose the system with ------ [number or unit weight of briquettes or tablets] of ------ [product name] per ------ [unit volume] of water in the system. (Name special container or name site in system.) Badly fouled systems must be cleaned before initial treatment.

(h) *Pulp and papermill water systems.* (1) Control of bacteria and fungi in pulp, paper and paperboard mill systems means to stabilize the microbial population at such a level that slimes will not interfere with system efficiency or product quality.

(2) Broad claims for use, such as microbicide, microbiostat, slimicide or microorganism control are acceptable only when modified by naming the types of organisms (bacteria, fungi) for which control is intended. Such terms as biocide or biostat are unacceptable where only microorganism control is to be construed.

(3) Cidal claims are acceptable only where tests demonstrate that the product effectively reduces the number of living bacteria and fungi. Static claims are to be made for products where tests demonstrate that they provide temporary control or growth suppression. The claim for a type of activity (cidal or static) must be related to each particular use claimed on the labeling. Examples include: Microbicide for use in pulp, paper and paperboard mill systems to reduce the number of living bacteria and fungi; and microbiostat (microorganism control) to control the growth of bacteria and fungi in paper machine systems.

(4) Directions for general microbial control must include, but are not limited to, the following:

(i) Where in the system the product is to be applied; for example, at a point in the system (fan pump, beater, pulp chest) where it will be uniformly mixed.

(ii) The timing of application; for example, continuously or intermittently for a certain number of hours per day or per shift.

(iii) The dosage to be applied. The dosage rate for solid products must be specified as weight of product per ton (dry basis) of pulp or paper produced. Labels of products formed into tablets, briquettes or other uniformly sized shapes must state the average weight of each unit. If the dosage rate for liquid products is stated on a fluid volume basis, the product weight per gallon must be shown on the application. If, however, liquid products are to be added on a

weight basis, the product weight per gallon must be shown on the label.

(iv) The method of application and pattern of use. In a mill system, dosage range depends on the severity and location of contamination. Methods of application are described as slug, intermittent or continuous.

(A) *Slug method.* Slug treatment, the addition of a specified dose of a pesticide to the system at one time, is intended to provide a high concentration of the toxicant for a short term. Slug treatments may be repeated at stated intervals, usually once a day or less frequently. Example of direction for use:

Apply ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ton (dry basis) of pulp or paper produced, as a slug dose. If needed, repeat ------ [stated interval]. Badly fouled systems should be cleaned before initial treatment.

(B) *Intermittent feed method.* Treatment is on a semicontinuous basis; for example, for 2 hr during each 8-hr shift. Directions must stipulate the dosage range to be employed, the usual time interval between doses, and the timing of application. The label must not claim maintenance of any particular pesticide concentration. Example of direction for use:

Apply ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ton (dry basis) of pulp or paper, for ------ [hr] every ------ [hr]. Badly fouled systems must be cleaned before initial treatment.

(C) *Continuous feed method.* Treatment is on an uninterrupted basis to provide a sustained concentration. The label should specify a dosage range that usually is somewhat lower than that for intermittent feed. In many cases, intermittent feeding may be used to achieve initial control, followed by lower-dosage continuous feeding to maintain control. Examples of directions for use:

Apply ------ to ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ton (dry basis) of pulp or paper produced on a continuous basis. Badly fouled systems must be cleaned before initial treatment.

Apply ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ton (dry basis) of pulp or paper produced for ------ [hr] every ------ [hr]. This regimen may be repeated until the desired control is achieved. When control is accomplished, apply ------ [fluid volume or unit weight] of ------ [product name or active ingredient] per ton (dry basis) of pulp or paper produced on a continuous basis. Badly fouled systems must be cleared before initial treatment.

(D) *Briquettes or Tablets.* These may be considered either a slug or intermittent method of application depending on the rate of dissolution. Data must be submitted showing the rates of dissolution of the product in water at various temperatures and flow rates. Example of direction for use:

Slug dose the system with ------ [number or unit weight of briquettes or tablets] of ------ [product name] per ton (dry basis) of pulp or paper produced. (Name special container and/or site in systems where the product is to be applied, so that mixing and distribution is uniform.) Badly fouled systems must be cleaned before initial treatment.

(i) *Antifouling Paints.* Label directions should prescribe the number of coats, the time interval between coats, and the time interval between the last coat and launching, when these factors are essential to the performance of the product. Currently it is not necessary to claim specific pests to be controlled, but the intended broad group or groups (algae and/or marine fouling animals) should be stated.

§ 162.74 Specific comments on fungicides and nematicides.

(a) *Coatings.* Fungicides intended for the preservation of coatings are pesticide products (industrial fungicides) and must be registered before they are shipped to the coating's manufacturer. (See General Comments, Claims for Manufacturing Use Only and Claims as Preservatives.) Formulated coatings containing fungicides that are not considered pesticides are those which have been treated to protect the coating itself against the growth of fungi while in the can [§ 162.4(d)(2)].

(b) *Mildew control.* (1) The control of mildew in homes, institutions, buildings, and similar environments, or the protection of articles or materials used or stored in such environments, is a very important aspect of pesticide usage. Mildew is defined here as the growth of molds (simple plants classified as filamentous fungi) on various surfaces, such as walls and ceilings, materials and articles made of leather, fabrics, paper, and wood, often causing musty odors.

(2) Formulations intended to be used to control mildew must state this claim on the labeling and provide adequate directions for use; that is:

(i) Name the types of surfaces, articles or materials to be treated and, when appropriate, specify cleaning before treatment.

(ii) State the rate of dilution or the concentration to use, if applicable.

(iii) State the method, degree and extent of application (for example, spray surfaces until moist or apply to dampen).

(iv) State recommendation with respect to the need for repeating applications at specified intervals (as determined by tests of residual properties).

(3) Some claim words and phrases are so broad that they are subject to many interpretations. Some imply different types of activity. Also, certain formulations have residual properties; others do not. The position of the Environmental Protection Agency with respect to various claims is as follows:

(i) The term fungicide (denoting killing action) or fungistat (denoting inhibition of fungal growth), when unmodified with respect to specific claims intended, may be interpreted as a claim that a product is effective against both (A) pathogenic fungi, such as Trichophyton interdigitale, or athletes foot fungus, on environmental surfaces; and (B) nonpathogenic fungi that cause mildew. Different tests are required for the 2 groups.

(ii) Two major categories of mildew claims are recognized, based on the degree of effectiveness implied by the wording of the claims.

(A) Kill claims mean kills mold and mildew fungi in all stages. Some equivalent claims are fungicide and destroys musty odors at their source. Chemicals, such as chlorine-releasing compounds, isopropyl alcohol and other volatile materials, may kill fungi on contact but have no residual properties to protect treated surfaces from later infestations. The claim for these products should be modified by adding nonresidual after the action word; for example, destroys mildew (nonresidual) or kills mildew fungi (nonresidual). An alternative would be destroys or kills mildew on contact, or similar phraseology. For formulations which kill fungi and also leave an effective residue, the direction should state the time interval (determined by testing) before repeated application is needed.

(B) Prevent claims mean that the treatment will prohibit the growth of mold or mildew organisms and denotes a strong and lasting residual property for a stated period of time (determined by testing). Equivalent claims are mildewproof, stops mildew and prevents musty odors at their source.

(NOTE: the claim aids in preventing mildew growth is unacceptable; the claim aids in control, however, is acceptable.) Lesser claims than prevent claims are controls, inhibits growth, mildew resistant, minimizes mildew, retards mildew, fungistatic.

(iii) Where a claim for musty odors (mustiness) is unqualified as to the causal organisms, it is assumed to include the control of mildew growth, which is a source of such odors.

(iv) Volatile products—products such as paradichlorobenzene cakes that control mildew growth by releasing vapors—must specify: (A) A dosage rate in terms of amount per cubic foot of space, (B) that the space be tightly enclosed or sealed, and (C) that additional material be added when the treated space is opened or unsealed. A claim that is worded clearly as a deodorant action (that is, masks or neutralizes the odor without direct action on the causal organism) is not considered a pesticide claim.

(v) Mildewproof and mildew resistant are considered fungistatic claims. Unless stated otherwise, they imply long-lasting protection to treated articles or materials—months, year or for the life of the materials. Such claims must be supported by long term tests which compare untreated and treated test samples of the articles or materials that have been exposed to mildew organisms in a simulated tropical environment.

(vi) Removes mildew stains is not considered a pesticide claim.

(c) *Nematode Pest Claims.* Depending upon the results obtained in nematicide efficacy tests, nematode pest claims may either be general or specific in nature. Specific claims must include the com-

mon names, (lesion, root-knot, cyst, etc.) of the nematodes to be controlled; general claims need only specify "nematodes" or "plant-parasitic nematodes."

§ 162.75 Specific comments on invertebrate control agents.

(a) *Household insecticides.*—(1) *Nonresidual insecticides.* (i) These products are ordinarily marketed as solutions, emulsions, suspensions, or pressurized products and designed for use in undiluted form by the consumer. In a few cases, concentrated products requiring dilution are marketed. The products usually have a petroleum distillate base, together with such auxiliary solvents as may be necessary to keep the formulation as a solution under conditions of relatively low temperature. Methylated nephthalenes, methylated aromatic petroleum solvents, and methylene chloride are frequently used as auxiliary solvents, although methylene chloride is more common in pressurized products. Water is sometimes used in liquid formulations. The propellants commonly encountered are Propellant 11 (trichloro monofluoro methane) and Propellant 12 (dichloro difluoro methane). Propellant 12 may be used alone or in various proportions with Propellant 11, methylene chloride, or methyl chloroform. Specific considerations relating to directions for use and precautionary statements for these products are discussed below.

(ii) In all cases, the products' labeling should bear adequate directions for use against all of the insects named in the labeling. These products are usually recommended for use against a number of household insects, including houseflies, mosquitoes, roaches (water bugs), bed bugs, ants, carpet beetles, brown dog ticks, clothes moths, wasps, and hornets. They are not effective against all household insects, and claims for effectiveness against insects generally or all insects are unwarranted and should not be made. Also, these products are primarily contact insecticides and, to be effective, must hit or wet the individual insect with the spray mist. Thus, as customarily marketed, they are not effective against termites and cannot be relied upon to kill any insect which cannot be reached directly by the spray. This applies also to the eggs of many insects, which are often placed in inaccessible cracks or hidden surfaces.

(iii) Since the habits and life cycles of different insect pests vary considerably, the directions must, in each case, be adapted to the particular variety of insect, as enumerated below, which is causing annoyance and the type of structure or building in which the product is used.

(A) *Flies and mosquitoes.* Directions for use should provide for closing doors and windows and thoroughly spraying all parts of the room, particularly toward the ceiling, so as to fill the room with a fine mist. The room should be kept closed for 10 to 15 minutes and the fallen insects should be swept up and destroyed. When strong formulations containing substantial amounts of rapidly acting paralytic agents are used, however, it is simply necessary to ascertain that the various insects have been thoroughly enveloped in the spray mist. Pressurized aerosol formulations, pressurized sprays and liquid products may be used in a similar manner. Dosages of aerosol and pressurized formulations are sometimes expressed in terms of seconds of discharge, with appropriate adjustments for low and high delivery rate dispensers. These dosages usually are in the range of 4 to 5 g of aerosol mixture in mist form per 1000 cu ft of space. The labeling should contain cautions to protect food and food utensils from contamination and to thoroughly clean any utensils, silverware, or dishes that might be contaminated.

(B) *Household ants and roaches.* The directions for use should provide for thoroughly spraying all parts of the room suspected of harboring these pests, especially cracks and hidden surfaces around sinks or food storage areas where the insects may be hiding. In all cases the insects must be contacted directly with the spray. Treatment around doors and windows is desirable in directions for use against ants. Pressurized formulations may be used, but since liberal application is essential, small pressurized dispensers may not give as good results in some cases. Repeated applications should be specified in all cases. The labeling should also contain cautions to protect food and food utensils from contamination and to thoroughly clean any utensils, silverware or dishes that might be contaminated.

(C) *Bed bugs.* The directions for use should provide for thoroughly spraying the bed, springs, and mattress, as well as the baseboards and wall cracks about the bedroom. Repeated applications are usually necessary for good results against these pests.

(D) *Clothes moths and carpet beetles.* The directions for use should provide for cleaning all articles to be protected and for thorough spraying, particularly of seams and folds. The interior of trunks, closets, cupboards, and other storage containers should also be thoroughly sprayed. Unless the sprayed articles are to be stored immediately in moth-tight containers, the directions should provide for repeating treatment at least once a month. For upholstered furniture, the directions should provide for spraying the interior as well as the outer surfaces of the furniture, unless the furniture can be fumigated to kill any hidden infestation of these pests. Rugs and carpets that are to be treated may also be sprayed on both the top surfaces and the under sides. When carpet beetles are a serious problem, however, a residual-type insecticidal treatment is preferable. Pressurized products, including aerosols, are less suitable for moth and carpet beetle treatments, since small dispensers do not ordinarily permit the liberal application that is usually necessary for good results.

(E) *Fleas and brown dog ticks in buildings.* Directions for use should provide for liberal applications to floor areas, cracks and crevices, sleeping quarters of animals, behind pictures, and wherever these insects are suspected of harboring. Liberal and repeated applications directly to the individual pests are desirable in all cases.

(F) *Mosquitoes and small flying insects outdoors.* Liquid and pressurized products can often be used effectively as mist spray applications for tall grass and shrubbery and around lawns where these pests may hover or harbor. This usage is suitable only in still air and requires frequent reapplication to kill additional insects that may be drifting into the area. It is not suitable for coping with any large influx of insects. Since many of these formulations are phytotoxic, care should be exercised to avoid wetting vegetation, and only mist spray application should be directed.

(G) *Hornets and wasps.* The directions for use should give instructions to spray the nests in the evening after the insects have congregated and are at rest.

(2) *Residual insecticides.* (i) These products are ordinarily marketed as oil solutions, emulsions, suspensions, powders, or pressurized products. Some are designed for use in undiluted form by the consumer; others are concentrated products that require various degrees of dilution. The products usually have a petroleum distillate base, together with such auxiliary solvents as may be necessary to keep the formulation as a solution under conditions of relatively low temperature. Methylated naphthalenes, methylated aromatic petroleum solvents, methylene chloride and methyl chloroform are frequently used as auxiliary solvents, although the last two are more common in pressurized products than in liquid formulations. Water is sometimes used in the liquid formulations. The propellants commonly encountered are Propellant 11 (trichloro monofluoro methane), Propellant 12 (dichloro difluoro methane), nitrogen, carbon dioxide, nitrous oxide, and certain hydrocarbons, such as propane. Propellant 12 is used alone or in various proportions with Propellant 11, or with methylene chloride or methyl chloroform together with small amounts of propane. Pressurized products of this class usually contain lower levels of propellants than are commonly encountered in the nonresidual-type products discussed above. Mothproofing treatments of woolens and fabrics to impregnate them with a protective chemical residue are excluded in this discussion, since they usually entail professional application. Specific considerations relating to directions for use and precautionary statements for these products are discussed below.

(ii) When general application is specified in the directions for use, the residual treatment may be applied to as many areas of the infested premises and to as large a proportion of the available surface as may be considered necessary for effective pest control. When usage is limited to "spot application," much greater attention should be given to limiting the treatment to those surfaces where pests have been seen or where they are suspected of hiding. Such treatments would normally be limited to a maximum of 20 percent of the floor and lower wall surfaces, including shelving and cabinets.

(iii) Many of the formulations are not acceptable for mist spray application in the air and care should be exercised to apply the products as relatively coarse wet sprays which will adhere to treated surfaces. This is especially important in the case of pressurized products.

(iv) Claims that residual insecticidal value will last for any particular period of time are usually undesirable because of the variable conditions under which the products are used. It may be stated that the product is a residual insecticide, but the labeling should emphasize the need for repeated applications as often as necessary, since it is not possible to anticipate all of the conditions of use, types of surfaces to be treated, and cleaning operations on treated surfaces.

(v) In all cases the products' labeling should bear adequate directions for use against all of the insects named in the labeling. These products are commonly recommended for the control of roaches (water bugs), houseflies, mosquitoes, silverfish, spiders, bed bugs, ants, carpet beetles, scorpions, fleas, brown dog ticks, crickets, wasps, hornets, earwigs and box-elder bugs. They are *not* effective against all household insects and claims for effectiveness against insects generally or all insects are unwarranted and should not be made.

(vi) Since the habits and life cycles of different pests vary considerably, the directions must, in each case, be adapted to the particular kind of insect, as enumerated below, which is causing annoyance and to the type of structure or building in which the product is used.

(A) *Flies and mosquitoes.* Directions for use should provide for thorough treatment of surfaces where these insects are known to alight or congregate. Emphasis should be placed on spraying or applying powders around doors, windows and porches. The labeling should contain cautions to protect food and food utensils from contamination and to thoroughly clean any utensils, silverware, or dishes that might be contaminated.

(B) *Household ants, roaches, silverfish, crickets, earwigs and box-elder bugs.* The directions for use should provide for thoroughly spraying or distributing the product into parts of the room suspected of harboring these pests, especially cracks and hidden surfaces around sinks or food storage areas where these insects may be hiding. Thorough treatment around doors and windows is desirable in directions for use against ants. Spraying around baseboards, windows, door frames, bookcases, and storage areas is usually desirable in directions for use against silverfish, crickets, earwigs, and box-elder bugs. The labeling should contain cautions to protect food and food utensils from contamination and to thoroughly clean any utensils, silverware, or dishes that might be contaminated.

(C) *Bed bugs.* The directions for use should provide for thoroughly spraying the bed, springs, and mattress, as well as the baseboards and wall cracks about the bedroom.

(D) *Carpet beetles.* The directions for use should provide for thorough application along baseboards, edges of carpeting, under furniture, under carpeting and rugs, and in closets and shelving where these insects are seen or suspected.

(E) *Fleas and brown dog ticks in buildings.* Directions for use should provide for applications to floor areas, cracks and crevices, sleeping quarters of animals, behind pictures and wherever presence of these insects is suspected. Liberal and repeated applications to the infested areas are desirable in all cases. Spot applications are not suitable for the control of fleas.

(F) *Spiders and scorpions.* Directions for use should provide for thorough wet spraying or dusting around windows, doors, baseboards, and crawl spaces, where these pests may occur.

(G) *Pantry pests.* Saw-toothed grain beetle, confused flour beetle, rice weevil, cigarette beetle, drug-store beetle, and Indian-meal moth are included in this category. Although cleanliness and good housekeeping are basic to preventing losses because of these pests, insecticides may sometimes be used carefully to advantage. Directions for use should provide for the removal of all packages of food and for the treatment of the surfaces of the shelves and the interior of cupboards with a residual spray. The spray must be allowed to dry before the packages are replaced on the shelf. Shelf paper or foil should be used to prevent contact of food or utensils with sprayed surfaces. It should be emphasized that this type treatment is of only supplemental value, since it does not affect the insects breeding inside the packages. Infested food packages should be destroyed or fumigated.

(H) *Hornets and wasps.* The directions for use should give instructions to spray the nests in the evening after the insects have congregated and are at rest.

(3) *Mosquito Abatement.* Label directions on mosquito coils should be limited to repelling mosquitoes on patios, porches, or similar confined outdoor areas where there is little or no breeze. Labels should instruct users to burn more than one candle or torch to provide sufficient repellent smoke or vapor to be effective.

(b) *Food Handling establishments.* (1) Nonresidual space and contact insecticides with labels including directions for use in food handling establishments must bear the following or similar caution:

When using the product in food handling establishments, apply only when the facility is not in operation. Food should be removed or covered during treatment. All food processing surfaces should be covered during treatment or thoroughly cleaned before using.

(2) These products are applied to obtain insecticidal effects only during the time of treatment. Space treatment is the dispersal of insecticides into the air by foggers, misters, aerosol devices or vapor dispensers to control flying insects and exposed crawling insects; contact treatment is the application of a wet spray.

(3) Some bait-type products may be used in food areas during periods when operations are shut down and all food and food handling surfaces are well protected or removed. Labeling should state that all bait containers must be removed and that cleaning procedures are required before operations are resumed.

(c) *Farm structures.* Label directions must specify the areas of treatment and the insects to be controlled. Directions on products intended to control stable flies should limit the sites of application to outdoor surfaces, since these insects rarely go inside enclosed structures. Labels must emphasize the following precautions, if pertinent.

(1) Do not contaminate food or feed products, drinking fountains, litters and troughs.

(2) Ventilate before allowing animals to reoccupy treated premises.

(3) Do not use in milk processing rooms, milk houses or milk storage rooms.

(4) Do not use in houses.

(5) Do not use on animals.

(6) Do not spray freshly whitewashed surfaces.

(7) Do not store or apply to surfaces within reach of children.

§ 162.76 Specific comments on vertebrate control agents.

The labeling submitted for registration must precisely identify the species of vertebrate pests against which the product is directed. Broad terms, such as rodents or nuisance birds, are unacceptable, unless the supporting research data actually includes representatives from all the principal groups. Even such terms as house rats and mice are unacceptable unless all 3 principal species, the brown rat (*Rattus norvegicus*), the roof rat (*Rattus rattus*), and the house mouse (*Mus musculus*), have been included in carefully controlled experimental studies.

CHEMISTRY

§ 162.77 Overview.

The following topics relate to data necessary for the general evaluation and registration of products and individual ingredients. All raw research data, such as gas chromatograms, photographs of thin-layer chromatograms and scintillation counter printouts, should be retained and available for 5 years, should the Office of Pesticide Programs request their submission. Also, all data placed on magnetic computer tape, such as total ion current chromatograms and mass spectra of significant components within a total ion current chromatogram, should be available for replay.

§ 162.78 General product chemistry.

Information related to general product chemistry also appears in § 162.10, Labeling Requirements—Net Contents, Ingredient Statement and Precautionary Statements.

(a) *Basic manufacturing process.* Information on the basic manufacturing process and on the purity of starting and intermediate materials should be submitted by the basic manufacturer with registration applications and petitions for tolerances. Other registrants may refer to the source of the technical chemical used in their product. Purity levels of such materials can vary considerably; significant changes in the materials or production methods also can introduce new types or concentrations of

impurities. These purity levels or changes can affect the performance or safety of the final product. A description of quality control procedures is required for disinfectant-type products and may be requested for other products where an impurity might induce an unreasonable adverse effect or affect the efficacy of the product.

(b) *Information required for active and inert ingredients.* The chemical composition of each pesticide, including both active and inert ingredients, must be submitted in a confidential statement or formula. (See Registration Procedures for a sample form and specific instructions for completing it.)

(2) In determining whether an ingredient is active or inert, the Administrator shall consider:

(i) The ingredient's capability by itself, when used as directed, to effectively control the target pest or perform as a plant regulator, defoliant, or desiccant.

(ii) The ingredient's presence in the product in a sufficient amount to add materially to its effectiveness.

(iii) The ingredient's influence on the activity of the principal active ingredient. The Agency may require that an ingredient be designated an active ingredient if it sufficiently increases the effectiveness of the pesticide to warrant such action.

(3) Unless they have pesticidal efficacy of their own, inert ingredients would include the following types of ingredients: Solvents, such as water; baits such as sugars, starches, and meat scraps; dust carriers, such as talc and clay; fillers; wetting and spreading agents; propellants in aerosol dispensers; and emulsifiers. These ingredients are not considered active ingredients although their use is necessary in the practical application of the pesticide.

(4) Active Ingredients: Chemical evaluation requires that certain basic information be available for each pesticide chemical. Although not usually required for established pesticide chemicals, it must be furnished with applications for registration of products containing any ingredient(s) not previously registered. This information includes, but is not limited to, the following:

(i) The common name, if established.
(ii) A well defined chemical name (Chemical Abstracts nomenclature).
(iii) Trade name.
(iv) Structural formula.
(v) Melting point.
(vi) Boiling point.
(vii) Vapor pressure.
(viii) Density or specific gravity.
(ix) Hydrolysis rate.
(x) Solubility in various solvents.
(xi) Dissociation constants (pKa or pKb).
(xii) Stability.
(xiii) Physical state.
(xiv) Color.
(xv) Odor.
(xvi) Composition of the technical product giving the names and percentages of the impurities.

(5) Inert ingredients: Although data requirements generally apply either to the individual active ingredients or to the entire formulation, if there is reason to believe that an inert ingredient may present problems of safety, environmental contamination or residue in crops, or if registration is requested for a formulation containing an inert ingredient not previously registered, some or all of the information required for active ingredients will be required for inert ingredients as well. As examples: The solvent dimethyl sulfoxide is restricted to a maximum of 15 percent of a formulation because it can penetrate the skin and injure the user; polychlorinated biphenyls and polychlorinated terphenyls cannot be used because they are very persistent in the environment and may detrimentally affect wildlife.

(6) Establishing a Common Name: If an ingredient has no common name, the Administrator may after due notice establish a common name, first considering names proposed by the applicant, registrant, or primary producers of the chemical. Names proposed by others also may be considered. Proposed names should be appropriate for the information and protection of the public. A simple chemical name may serve as a common name; a proprietary name or trademark will not be acceptable. The person proposing a common name will be responsible for ensuring that it does not infringe on a valid trademark, patent, or copyright. Names may be established by application to the K62 Committee of the American National Standards Institute, 1430 Broadway, New York, New York 10018. The Agency has available a compilation of all acceptable common and chemical names.[3]

(c) *Assay and impurity assessment for technical products.* (1) Analyses and the methods employed are required for the principal component and for the significant technical impurities in each pesticide chemical. These analytical techniques usually will be combinations of such methods as thin-layer chromatography, ion-exchange separation, gel filtration fractionation, liquid chromatography, gas chromatography, infrared spectroscopy and mass spectrometry. Limits of variation for each significant impurity should be provided for normal production batches of the pesticide chemical. Specifications for baseline levels of the various impurities can be established from these data.

(2) In addition to the preceding requirements, for technical ingredients not previously registered, a 2–g sample of a purified analytical standard (of known composition) of the pesticide chemical and a 10–20 g sample of a typical batch of the technical material may be required.

(d) *Information required for formulations.* Registration applications should also include the following information about the formulation:

(1) Miscibility in various solvents.
(2) If applicable, the pH, boiling point, flashpoint, specific gravity, viscosity and vapor pressure.
(3) Explosive characteristics, if any.
(4) Corrosion hazards.
(5) Capability of acting as an oxidizing or a reducing agent.
(6) For liquids, the weight of the active ingredient(s) per gallon.

(e) *Storage stability.* Data, usually chemical analysis, covering a period of at least 1 yr should be submitted to show storage stability. Normally, the product, as commercially packaged, must be stable for as long as it would be expected to remain in channels of trade. As a general rule, if during 1 yr, the percentage of any predominant active ingredient declines more than 5 percent from the original value, thereby becoming less than the percentage stated on the label, this would be significant. If significant deterioration does occur during normal storage periods, further justification for the product, including additional toxicity and performance testing, may be required, and the provisions of § 162.10(g)(6) concerning label warnings of deterioration will apply. Accelerated aging tests can sometimes be accepted on an interim basis, but shelf life studies under conditions usually encountered in transportation and storage shall be completed and the data submitted within 2 yr after registration.

(f) *Pesticides on tobacco.* (1) Tobacco is not listed as a raw agricultural commodity requiring the establishment of a tolerance. For registration purposes, the following residue considerations for products for use on tobacco include a total residue profile in the tobacco and the smoke. In addition to the parent compound, this profile includes polar and nonpolar plant metabolites, translocated degradation products from soil, and photodegradation products. Radiochemical techniques will normally be required; if significant residues are detected, analytical methods should be developed to characterize the residual products and test data should be submitted.

(2) If the pesticide use pattern represents a foliar treatment or application to soil, a study on followup crop tobacco may be required. This study should compile adequate data to show conclusively the level of residue likely to result from the directed use of the product, including:

(i) Residues on green, freshly harvested tobacco. Proceed with step 2 only if residues are detected.

(ii) Residues on cured or dried tobacco. Proceed with step 3 only if residues are detected.

(iii) Residues in smoke from treated tobacco. Proceed with step 4 only if residues are detected in smoke.

(iv) Identify significant pyrolysis products, and conduct subacute inhalation studies. (See Hazard Evaluation.)

§ 162.79 Environmental chemistry.

(a) *Pesticide fate and movement in soil—(1) Edaphic and climatic descriptions.* (i) The soil description should fit

[3] Robert L. Caswell, David E. Johnson, and Conrad Fleck, Acceptable Common Names and Chemical Names for the Ingredient Statement on Pesticide Labels, 2nd ed. (Washington, D.C.: U.S. Environmental Protection Agency, 1972).

into a common textural classification.[4] The Soil Conservation Service (USDA) field office serving the county may be consulted in locating a representative site and in classifying the soil.

(ii) The following physical and chemical properties of the surface soil should be determined: Organic carbon, cation exchange capacity (at pH 7), pH, bulk density, and water retentivity at ⅓ bar. Acceptable methods would include those described in the Soil Conservation Service, Soil Survey Investigations Report No. 1, or in the American Society of Agronomy Monograph No. 9. (See Appendix Part VI.) A complete report of the studies conducted, including a description of or reference to the actual method used, should be submitted.

(iii) Field studies with pesticides should also be supported by the following climatological and geographic information as appropriate:

(A) Sufficient descriptive data to locate the field plot(s) within a county or a parish.

(B) Amount of rainfall and/or irrigation water accumulative to each sampling.

(C) Soil and/or air temperature data from the State climatic division within which the field studies were performed.[5]

(2) *Soil Metabolism Studies.* (i) These studies should be designed to provide basic information on the rate, type and degree of degradation of the parent pesticide and its transformation products, including bound residues. Since knowledge of all major products formed is essential, studies with radiolabeled pesticides will be required. Radiolabeling should be in a strategic portion of the molecule that will remain in the significant degradation products. Usually, but not always, labeling will involve an aromatic ring. Some highly complex pesticides will require radiolabeling at several points in the molecule to assure adequate coverage of all chemical transformations. Greenhouse studies under controlled conditions are acceptable.

(ii) Rapid loss of carbon dioxide is not, in itself, satisfactory evidence of a product's safety. Although the major metabolic pathway may take this route, other routes may lead to the formation of small, but significant, amounts of by-products. Sufficient data should be collected by periodic analysis to allow graphic plotting of the amount of parent compound until 10 percent or less remains. The data should clearly show the formation and decline rate of significant degradation products. Proper isomeric designations and structural formulas should be provided for each compound and significant degradation product.

(iii) As a result of the studies, clearly defined and validated analytical procedures for the separation and identification of degradation products and polar and nonpolar metabolites should be available. Of special importance are mobility (Rf) values on thin-layer chromatograms and relative retention times by gas chromatography. Studies should be supported by a complete list of all model compounds synthesized for use in the metabolism study or for any other purpose related to registration. In addition to this listing, details of method of synthesis and analytical method parameters should be furnished. Distribution coefficients (p-values) will be required on all significant degradation products. (See Appendix Part VI.)

(iv) Specific activity of the radiolabel should be capable of tracing metabolites down to 10 ppb in the soil. Counting levels should be at least twice that of the normal background level; or, if not, counting times should be long enough to provide a high degree of statistical significance. Critical counting data should always be obtained after total combustion to minimize quenching effects.

(v) Basic soil metabolism studies should be performed under aerobic conditions. For standardization and comparison, a soil with sandy loam, loam, or silt loam surface texture is preferred. (See Appendix Part VI.)

(vi) An additional abbreviated test using a fine textured surface soil is desirable to determine if significantly different levels of bound residues occur in such soils and to provide comparative qualitative data on metabolic products in a significantly different soil type. (See Appendix Part VI.) Residues remaining after exhaustive extraction with nonpolar solvents followed by polar solvents will be considered bound residues. An interim definition of a significant quantity of bound residue has been established as any amount of unextractable residue, greater than 10 percent expressed as parent compound, remaining 1 yr after a single treatment.

(vii) Data showing that a residue is strongly bound justifies continued experimental inquiry into the nature and possible significance of the binding mechanisms; potential for release; and slow long-term interactions of several different chemical substances. Therefore, the characterization of a residue that is strongly bound will require data related to the following questions.

(A) Will bound residues continue to increase with annual application of a pesticide to the same soil?

(B) Will a plateau be reached?

(C) What is the dissipation rate of bound residues?

(D) Can bound residues be taken up by subsequent crops growing in the soil?

(vii) In addition, tests to provide data relating to these questions may be needed:

(A) What is the nature of the bound products?

(B) In what constituent of the soil are they bound?

(C) What is the binding capacity of various kinds of soils?

(D) Do various pesticide degradation products compete for available binding sites? (For example, will phenols displace amines, or vice versa?)

(E) If the soil particles reach an aquatic environment, what pesticide fragments are released and over what period of time?

(ix) In addition to the aerobic soil metabolism studies, an anaerobic soil metabolism study will be required. (See Appendix Part VI.)

(3) *Soil persistence studies.* (i) Soil persistence data derived under actual use conditions are required in the main geographic and/or climatological areas for which a registration is proposed. Normally 4 sampling sites selected to represent ranges of the weather and soil elements will be adequate. Suitable classifications into soil regions include the 20 land resource regions described by the USDA.[6] Additional considerations should include the amount and the time distribution of precipitation and irrigation, mean air temperature for the growing season, and mean annual freeze-free periods.

(ii) Samples should be taken using statistically valid sampling schemes over a sufficient timeframe to allow a definitive assessment of the degradation profile. Besides the parent pesticide moiety, persistent degradation products of significant quantities should be determined. Suggested sampling times (after the last treatment) would include 0 days, 1 wk, 2 wk, 1 mo, 3 mo, 6 mo, 1 yr, and 18 mo. Sampling depth should be at least 0 to 6 in.; if the pesticide leaches, deeper sampling profiles may be necessary. The sampling should clearly encompass the entire vertical distribution of the parent pesticide and its major degradation products.

(iii) When possible, each site utilized should represent a major soil series in the particular geographic region. When 3 or more land resource regions are represented, however, at least one of the more northerly regions should be represented by a fine-textured surface soil (finer than silt loam).

(iv) For many pesticides with low-to-moderate persistence (half degradation times of less than 6 mo), the preceding studies will provide adequate data on the degradation profile under typical field conditions. Some agricultural pesticides, however, that are not incorporated directly into the soil, such as those applied to foliage or used in preemergence weed control, may yield ambiguous soil persistence data. In these cases, supplemental soil persistence studies in which the pesticide is incorporated directly into the upper soil horizons may be appropriate. (See Appendix Part VI.)

(v) If a pesticide or its degradation products are highly persistent, special long-term studies to determine buildup of these substances may be required. Usually, studies would entail selecting test fields and employing the maximum

[4] U.S. Department of Agriculture, *Soil Taxonomy*, Agricultural Handbook No. 436 (Washington, D.C.: U.S. Government Printing Office, in press).

[5] U.S. Department of Commerce, *Climatic Atlas of the United States* (Washington, D.C.: U.S. Government Printing Office, June 1968).

[6] U.S. Department of Agriculture, *Land Resource Regions and Major Land Resource Areas of the United States*, Agricultural Handbook No. 296 (Washington, D.C.: U.S. Government Printing Office, 1972).

allowable application rate and number of treatments (according to the label instructions) for a succession of growing seasons. Test areas would be selected for climatological conditions most favorable to stability of the parent compounds. These studies should continue until it is clearly demonstrated that a plateau level or equilibrium is reached; or the buildup due to repeated applications of the pesticide can be predicted.

(4) *Leaching studies.* Laboratory scale leaching studies on the parent compound and its soil degradation products will be required. If a high degree of mobility is observed for the pesticide or one of its degradation products, special field studies may be required. (See Appendix Part VI.)

(b) *Pesticide fate and movement in water.* Typical studies which may be required for a new pesticide product, including the parent compound, metabolites and any important degradation products, are summarized below. The appendix provides information on various tests that can be performed to obtain data.

(1) *Dissipation rate in distilled water.* These studies should include:

(i) Data on the rate of hydrolysis at acid, basic and neutral pH values. Test temperatures should span the range of water temperatures anticipated under actual pesticide use patterns.

(ii) Suitable photodegradation data at a pH where maximum stability has been noted.

(2) *Degradation in water containing suspended solids.* These studies should determine:

(i) The effect of suspended sediment under natural conditions. Studies should include qualitative and quantitative data on any major metabolite and degradation product.

(ii) Duration of biological activity, such as phytotoxicity.

(3) *Degradation studies in bottom sediments.* If residues accumulate in bottom sediments, dissipation curves should be determined in sediments representative of those likely to be encountered under normal conditions of use.

(4) *Translocation.* Laboratory and/or field studies on pesticide movements should include:

(i) Uptake of the pesticide and its breakdown products by roots and foliar surfaces of typical crops and aquatic plants that would be receiving some or all of their water from the aquatic source.

(ii) Residue levels in irrigated, flooded, or otherwise artificially watered food crops.

(5) *Livestock and poultry drinking water.* Studies should show levels of residues, if any, in milk, animal tissues and eggs in relation to the concentration of the pesticide in water.

(6) *Tests involving moving waters.* Data on rate and extent of movement in flowing water would normally be obtained in one or more of the following ways:

(i) Chemical analysis of water from selected points downstream from source of entry.

(ii) Bioassay of water from selected downstream points, using highly sensitive aquatic plant or animal organisms.

(iii) Chemical analysis of residue buildup in selected indicator organisms.

(c) *Pesticide residue studies.*—(1) *Fish and wildlife studies.* (i) Fish residue studies must be conducted to determine the amount of uptake of the pesticide and its major degradation products by fish, until a plateau has been reached and the rate of dissipation of any residue taken up by fish when placed in pesticide-free water. These studies are usually run in dynamic flow-through systems, but, with adequate controls, static tests are acceptable.

(ii) Normally, the fish residue requirements can only be met using radiochemicals. Detailed test procedures can only be defined by careful consideration of use patterns and a knowledge of the pesticide's degradation characteristics in various segments of the environment. Analytical determinations should include the relative amounts of extractable (polar and nonpolar) and tissue-bound residues.

(iii) Modified fish exposure studies involving pesticides which are strongly adsorbed on soil surfaces will be considered on a case-by-case basis.

(iv) In addition to the fish residue studies discussed above, additional studies on fish and other aquatic organisms may be required under exposure conditions simulating their natural habitat. Also, additional residue studies on terrestrial wildlife may be required depending on the proposed use pattern.

(2) *Crop uptake studies.* Studies on the uptake of pesticide residues in soil will be required for pesticides with use patterns proposed for croplands, or for noncroplands that may be converted for crop use.

(d) *Photodegradation studies.* Photodegradation studies on the pesticide will be required on surfaces, in water, and in air depending on the nature of the compound and its use pattern. (See Appendix Part VI.)

(e) *Volatilization studies.* Studies demonstrating the rate of volatilization of the pesticide under typical conditions of use may be required. (See Appendix Part VI.)

(f) *Microbiological studies.*—(1) *Effect of pesticides on microorganisms.* Integrated pest control operations often require the application of several different pesticides to the same area within a short timeframe. One or more of these pesticides, on reaching the soil, could significantly reduce or eliminate populations of nontarget soil microorganisms and thereby alter the biochemical characteristics of the soil. The alterations could affect soil fertility, or the degradation of some of the pesticides and result in increased persistence of a pesticide. Thus tests on soil microorganisms or relevant enzyme systems of natural soil populations will be required to assess the overall action of a pesticide.

(2) *Effect of microorganisms on pesticides.* In general biodegradability is a major factor affecting the persistence of pesticides. In aquatic sites, where such conditions as turbid water or anaerobic environments may prevent other means of decomposition, such as photolysis or oxidation, biodegradation assumes even greater importance. Because biodegradation relies extensively on the action of microorganisms, a qualitative and quantitative assessment of their role in the degradation of all pesticides likely to enter aquatic environments in significant quantities will be required. These pesticides would include aquatic herbicides, household pesticides and industrial microbicides.

Hazard Evaluation

§ 162.80 Overview.

(a) The basic purpose of FIFRA, as amended, is to ensure that pesticides used in the United States perform their intended functions without causing unreasonable adverse effects on man or the environment. This part sets forth Guidelines for collecting and reporting toxicity data which an applicant for the registration or reregistration of a product registered prior to the effective date of this Part 162 (FIFRA section 3 Regulations) must provide to the EPA pursuant to § 162.8. The data on toxicity enables EPA:

(1) To determine whether registration should be approved and whether the pesticide product should be classified for general use or restricted use according to the criteria of § 162.11.

(2) To determine whether the proposed labeling contains adequate precautions and warnings to protect pesticide users, and exposed humans, domestic animals, fish, and wildlife.

(3) To establish residue tolerance to protect consumers of exposed foods.

(4) To determine conditions under which farm workers can enter treated fields.

Adverse effects resulting from pesticide use depends upon the degree of exposure as well as the degree of toxicity and the type of toxic effect. The physical and chemical nature of the pesticide and its metabolites and degradation products are important determinants of potential hazard. The data requirements contained in Chemistry are essential in evaluating the hazard posed by a pesticide. The following sets forth for an applicant the toxicological data and reporting guidance for pesticide registration.

(c) The Appendix to accompany Hazard Evaluation contains test protocols for experimentation which EPA has determined are adequate for developing data to determine the hazards posed by the use of a pesticide. To meet registration requirements, any test procedure used by an applicant must meet the intent and reliability of the test procedures contained in the Appendix and conform to these Guidelines. If the applicant plans to use a procedure which significantly deviates from the test procedures, the applicant should consult with EPA to determine if the changed procedure satisfies the requirements of Registration Division. In these Guidelines, data are identified as either basic and required for all pesticide registrations or

conditional and required only under certain stated conditions. If an applicant contends that required data are not applicable to a specific product, he must submit a written statement to that effect and consult with EPA before embarking on a modified program of investigation. Data requirements set forth in these Guidelines and the Appendix will only be waived pursuant to § 162.8(a)(3), which provides:

An applicant for registration may submit written evidence that the composition, degradability, proposed patterns of use and such other chemical or physical properties of a specific pesticide or product relating to an evaluation of the effects on the environment or man are fundamentally different from the considerations made by the Agency in the establishment of the data requirements of the Registration Guidelines and that therefore some or all of the data requirements of the Registration Guidelines are inapplicable to the specific pesticide or product. After considering the evidence submitted by the applicant, and such other information as may be available to him, the Administrator will make a written finding with respect to whether such properties of the specific pesticide or product are fundamentally different from the considerations made by the Agency in establishing the data requirements of the Registration Guidelines. If the Administrator determines that such properties of the specific pesticide or product are fundamentally different from the considerations made by the Agency, he may waive a data requirement specified in the Registration Guidelines when he determines that the data so required is not necessary in order for him to determine whether such specific pesticide or product will generally cause unreasonable adverse effects on the environment or man.

(d) The pesticide used for toxicological testing must be the same chemically characterized product which is proposed to be or is commercially produced and used. Tests must be conducted at intervals to check for stability of the substance tested. It may be necessary to require additional animal studies to those prescribed below, based on the slope of the resulting dosage-response curve, the severity and duration of toxic signs and other appropriate observed effects. See also § 162.8(d).

(e) All toxicological and hazard evaluation studies must be done under the direction of qualified personnel, who are responsible for utilizing sound scientific experimental procedures adequately to determine a pesticide's toxicological hazard. Nothing in this part shall be construed as relieving the pesticide developer and researcher of that responsibility.

§ 162.81 Hazards to humans and domestic animals.

(a) This section outlines, first, data requirements for all new registrations. For those pesticides which have been registered before October 21, 1974, and which must be reregistered and classified under the mandate of the amended FIFRA, data requirements are somewhat modified. They are enumerated in a second topic.

(b) Data requirements for new registrations. (1) The objective of acute toxicity testing is the demonstration of the lethal dose of a pesticide for 50 percent of an animal group, expressed as a LD–50 or LC–50, and identification of the toxic signs consequent to a single exposure. The results of these procedures are used to:

(i) Demonstrate comparative mortality risk for determining the proper labeling and classification for each pesticide use;

(ii) Delineate specific toxic manifestations and mechanisms;

(iii) Develop appropriate clinical management techniques for persons exposed to a hazardous dose either from accident or misuse of the pesticide;

(iv) Determine the need for additional hazard studies, such as long-term chronic studies;

(v) Design dosing regimens and other aspects of subacute and chronic studies; and

(vi) determine whether the pesticide is to be subject to a rebuttable presumption against registration or presumptively classified for restricted or general use according to § 162.11.

(2) When the LD–50 or LC–50 is reported, it must be accompanied with calculations of the slope of the dose-response line, together with 95 percent confidence intervals for the LD–50 or LC–50 and the slope. For an acceptable method of computing LD–50's and confidence intervals, see footnote 7.

(3) Basic tests.

(i) *Acute Oral LD–50.* The single dose oral LD–50 is to be established on the basis of tests using 1 mammalian species, preferably the rat. A complete description of effects observed, including full details of behavior changes and gross pathological findings at post-mortem, must be reported. Tests are to be performed with both the technical grade material and the formulated product. In addition, this test must be performed on the product as diluted for use if the pesticide is used in domestic situations. This information is important for determinations regarding the classification of pesticides. It is not of practical value to administer pesticide doses beyond 5000 mg/kg in the acute oral or dermal LD–50 test. If sufficient members of animals have been used to establish the absence of toxic manifestations at this level, higher doses are unnecessary.

(ii) *Acute Dermal LD–50.* The single dose dermal LD–50 is to be established on the basis of tests of at least 1 mammalian species. The rabbit is the animal of choice, but the guinea pig and rat are also acceptable. Tests are to be performed with the technical grade material and the formulated product. As in the oral LD–50 computation, all results must be completely reported. In addition to determining an LD–50 and systemic effects, a well designed and executed test can also be used to determine local dermatologic effects, thus eliminating the need for an additional primary dermal irritation test.

(iii) *Acute Primary Dermal Irritation.* Primary dermal irritation is to be evaluated with the technical grade material and the formulated product on at least 1 mammalian species. The rabbit is the animal of choice, but the guinea pig and rat are also acceptable.

(iv) *Acute Primary Eye Irritation.* Primary eye irritation is to be evaluated with the technical grade material and the formulated product on at least 1 mammalian species. The rabbit is acceptable for this test.

(4) Conditional Tests.

(i) *Acute*—(A) *Inhalation LC–50.* When the physical and chemical nature of the technical grade product or the formulated product under condition of use results in a respirable dust, mist, gas, or vapor, an inhalation LC–50 must be determined. Tests are to be conducted on 1 mammalian species. The rat is the animal of choice.

(B) *Other routes.* Intravenous or intraperitoneal routes of administration may, on occasion, be needed to resolve questions raised by the oral, dermal, or inhalation studies. For example, if the extent of the gastro-intestinal absorption of a pesticide is uncertain, the acute intraperitoneal and/or intravenous toxicity of the chemical in the same species that was used to evaluate the acute oral toxicity may be requested.

(ii) *Subacute tests.* Subacute toxicity procedures are designed to determine the adverse effects of multiple exposure within less than ½ the lifetime of the organism. Such test procedures may be utilized to determine the general toxicity resulting from multiple exposures, as is usually the case with subacute feeding procedures, or they may be utilized to evaluate the potential of a pesticide to elicit specific reactions.

(A) *Subacute dermal.* Skin sensitization and subacute dermal data will be required if conditions of use may result in repeated skin contact (through dusting or bathing pets, impregnation of fabrics or leather, direct application to livestock, use as a swimming pool additive or insect repellant, etc. The guinea pig is the animal of choice for skin sensitization studies; the rabbit, the animal of choice for subacute dermal data. Tests must be performed with the technical grade product and the formulated product. Photosensitization studies should be performed if conditions of use may result in repeated skin contact and exposure to sunlight with pesticide products that contain coal tar derivatives, pyridine, anthracene or other related chemicals that are known to elicit photosensitivity reactions.

(B) *Subacute inhalation.* Inhalation studies on the formulated product will be required if conditions of use may result in repeated inhalation exposure. At least 1 mammalian species must be used; the rat is the animal of choice.

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[7] Weil, C. S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. *Biometrics*, 8: 249–263, or Litchfield, J. T. and Wilcoxon, E. 1949. A simplified method of evaluating dose-effect experiments. *Jour. Pharma. Exp. Therapy*. 96: 99–115.

(C) *Subacute oral.* Feeding studies will be required if use results in residues on food or feed or if significant oral exposure is expected, e.g. the swallowing of pesticides in swimming pool water. At least 2 mammalian species must be used, 1 a nonrodent, but excluding the rabbit.

(D) *Teratology.* Teratological studies are required on the technical grade product if the pesticide use may reasonable be expected to result in exposure to female humans, or if use may result in residues in food or feed. Teratological effects must be evaluated in tests using 1 mammalian species that possesses a hemochorial placenta, such as the rat, mouse, or non-human primate. Other species of proven value in this area, such as the dog, also may be used. In both this and reproduction studies, teratogenic effects must be distinguished from fetotoxic effects or generalized toxic effects on the parents.

(E) *Neurotoxicity.* Regardless of the route of exposure or the duration of the test, observations in experimental animals should be made for changes in behavior patterns, or other central nervous system effects. If the test compound is an organophosphate or carbamate pesticide or if it otherwise results in cholinesterase inhibition, neurotoxicity tests are required. The adult hen is acceptable for determining the effects of pesticides on the myelin sheath. The rat or dog is appropriate for demonstrating acetylcholinesterase inhibition.

(F) *Metabolism.* Metabolism studies are required if the pesticide use results in residues on food or feed. Depending upon the use and the nature of the residue on the raw agricultural commodities, 1 or 2 species should be studied. For example, if the pesticide is to be used in a manner likely to leave a residue in or on the feed of dairy cattle, the metabolism of the pesticide in the cow should be studied. Similarly, if the pesticide is to be used in a manner likely to leave residues in or on chicken feed, the metabolism of the pesticide in chickens should be studied. The rat and the dog are the usual laboratory species for extrapolating the metabolism of pesticides to man, however. The appendix provides information on testing pesticides in both ruminants and non-ruminants. Metabolism studies also may be required to define blood levels, tissue storage, detoxification mechanisms, or to resolve unusual toxicological problems.

(iii) *Chronic tests.* Chronic testing procedures determine the toxicological effects from multiple or continuous exposures over a period of at least ½ the lifetime of that organism. The purpose of these testing procedures is to establish a maximum exposure level that induces no toxicological effect and to determine the nature and degree of long-term toxic effects observed at various exposure levels.

(A) *Oncogenicity.* Oncogenic evaluation of the active ingredient will be required if the active ingredient(s), its metabolite(s), degradation product(s), or impurity(ies) contains a substance structurally related to a suspected or known oncogenic agent, produces hyperplasia or metaplasia; or if the pesticide use results in a residue on food or feed. Lifetime feeding studies are required in at least 2 mammalian species. The rat, mouse, and hamster are the animals of choice. One species used in the testing procedures must be the rat; the other species must be the most-sensitive species for that pesticide or chemical group, if such species has been established. For organochlorine pesticides, the mouse is the most-sensitive species and shall be the second species for testing. The most-sensitive species is generally determined in acute toxicity studies on a pesticide by pesticide basis; however, little information is available on most-sensitive species for lifetime feeding studies. As these species are identified, information will be provided in the Guidelines and Appendix.

(B) *Feeding.* Feeding studies are required if the pesticide use results in a residue on food or feed. At least 2 mammalian species must be used in these lifetime feeding evaluations. One species must be the rat; the other species will be determined by Registration Division. However, when a generally recognized most-sensitive species is appropriate for the particular pesticide, it will be prescribed. In most instances, life-time feeding studies also will provide data on oncogenicity that will be acceptable.

(C) *Reproduction.* Reproduction studies are required if the pesticide use results in a residue on food or feed. Evaluations must be performed on at least 1 mammalian species, using one of the same rodent species used in the feeding study. These studies will demonstrate whether abnormalities occur in parental activities from mating through lactation, and in progeny growth and development from inception through maturity.

(D) *Other Chronic Tests.* Experiments involving chronic exposures of experimental animals, usually via the oral route, shall also include observations of the effects of the pesticide on: (*1*) Hematopoiesis, (*2*) endocrine systems, and (*3*) histopathology of various tissues and organs, particularly the liver and kidney.

(iv) *Special Studies.* The following additional studies may be required under the special conditions indicated:

(A) *Mutagenicity.* Evaluation of the mutagenic potential of the active ingredients of a pesticide product will be required if the pesticide's use characteristics or chemical structure meet any of the following conditions:

(*1*) The pesticide requires a tolerance or an exemption from the requirement to obtain a tolerance;

(*2*) The active ingredient(s), its metabolite(s), degradation product(s) or impurity(ies) contains a substance structurally related to a suspected or known carcinogenic or mutagenic agent;

(*3*) The pesticide use pattern is likely to involve significant inhalation or dermal exposure to significant population groups.

The evaluation of mutagenic potential shall be conducted on *in vivo* mammalian test systems. The Appendix provides references on *in vivo* cytogenetics, a heritable translocation test and a specific locus test. The Agency realizes that, because of the special test animal and other requirements posed by these tests, they cannot be carried out by many laboratories at the present time. With current laboratory capacity, therefore, careful scheduling of mutagenic tests on mammalian test systems is necessary. The Agency believes, however, that in the near future when the demand for such tests becomes apparent, both academic and industrial laboratories will equip themselves to handle the workload. Government, academic and industrial researchers are also assessing the utility of tests on indicator organisms other than whole mammals for mutagenic evaluation and how they correlate with the mammalian experience. The Agency is closely following this assessment and, at the time when the reliability and effectiveness of nonmammalian tests have been established, will appropriately revise the requirements for mutagenic evaluation. During this evaluation phase, however, results of nonmammalian tests that indicate positive mutagenic potential may come to the attention of the Administrator. Such positive findings will not, in themselves, necessarily form the basis for a decision by the Administrator to deny the registration of a pesticide product. The Administrator will decide on a case-by-case basis whether the evidence warrants that a mutagenic or oncogenic evaluation be performed in mammalian test systems.

(B) *Potentiation.* If there is a reasonable possibility that the toxic effects of a pesticide could be potentiated by another chemical in the formulation or by another pesticide product with which it may be used, potentiation studies will be required.

(C) *Reentry.* Foliar residue and other studies measuring the exposure of persons reentering an area treated with pesticides will be required for cholinesterase inhibiting pesticides. These studies shall be designed to provide sufficient data to establish satisfactory precautionary statements. Characteristics of the pesticide which influence the requirement for reentry data include: (*1*) high acute toxicity by the oral, dermal, or inhalation routes of exposure, (*2*) long half-life of the chemical, taking into account volatility, so that it may be expected to be present in sufficient quantities to present a hazard to the worker when he enters the field, and (*3*) strong sensitizing activity in human or laboratory animals.

(D) *Other Studies.* When appropriate, additional studies based upon similarity of the chemical structure between the test compound and those known to produce specific toxic effects may be required. Studies may also be required to determine reversibility of effects found after subacute feeding.

(c) *Data requirements for reregistration.* If the toxicity testing data previously submitted to EPA conforms to the requirements of these Guidelines and § 162.8, no acute toxicity testing for evaluation of hazards to man are required for reregistering pesticides that are currently registered with EPA. The category

of toxicity rating that has been assigned to each pesticide will be used for classification, under the provision of § 162.11, where additional toxicity testing is not required. The applicant for reregistration must submit or have submitted the following data as specified:

(1) Teratogenic evaluation of the technical grade product in a mammalian test system is required if the pesticide use may reasonably be expected to result in exposure to female humans or if use results in residues in food or feed.

(2) Oncogenic evaluation of the technical grade product will be required if: (i) The active ingredient(s), its metabolite(s), degradation product(s) or impurity(ies), contains within its structure a chemical moiety(ies) typically found in compounds which initiate oncogenesis in mammalian test systems; or (ii) the pesticide needs a tolerance or an exemption from the requirement to obtain a tolerance.

(3) If the technical grade product proves to be oncogenic in a mammalian test system and if the product is used in residences, or enclosed working spaces, or their immediate vicinity, a mutagenic evaluation may be requested.

(4) Chronic feeding studies will be required for pesticides which need a tolerance or an exemption from the requirement to obtain a tolerance; or pesticides for use in residences, working spaces, or their immediate vicinity. Such chronic feeding studies shall include studies of effects on the central nervous system and hematopoietic system, and historological changes in the liver, kidney, and reproductive systems (male and female).

(5) Reproduction studies will be required for pesticides which need a tolerance or an exemption from the requirement to obtain a tolerance. Such reproduction studies shall consist of three-generation investigations.

(6) Foliar residue and other studies measuring the exposure of persons reentering an area treated with pesticides will be required for cholinesterase inhibiting pesticides. These studies will be designed to provide sufficient data to establish satisfactory precautionary statements.

The animal species used to develop needed data for reregistration are the same as specified above for new registrations.

(d) *Data requirements for five-year renewal of registrations.* Under the provisions of section 6(a) of FIFRA as amended, a pesticide registration must be renewed at least every five years. Before the end of that period, the registrant must request that the registration be continued in effect. Pursuant to section 6(a) of the Act and § 162.6 governing the registration of pesticides, the registration shall be continued in effect only upon determination that the registration complies with all requirements of the Act, the current regulations, and guidelines promulgated thereunder. If new data requirements have been added for registration during the registration period, the registration will not be renewed unless the registrant has submitted such additional data to EPA. The Guidelines will be amended from time to time to reflect these additional data requirements.

§ 162.82 Hazards to fish and wildlife.

(a) Acute and subacute toxicities are the initial criteria by which potential hazard to fish and wildlife is evaluated. Data must be secured for all pesticides that will be used outdoors or that may contaminate water or other environmental resources. Data on the species listed below are normally sufficient for a general appraisal of the toxicity of pesticides to fish and wildlife.

(b) In evaluating the potential hazard posed by a proposed pesticide use, toxicity data will be considered together with such other factors as:

(1) The chemical and physical characteristics of the pesticide.

(2) The amount of pesticide applied per unit area and/or unit time.

(3) The likely degree of contamination of different environmental components under proposed conditions of use.

(4) The various species likely to be exposed to the pesticide.

(5) The routes by which organisms contact the pesticide and the likelihood of exposure in different components of the environment.

(6) The persistence of the pesticide or its biologically active metabolites and transferral between environmental components.

(7) The degree of bioaccumulation of the pesticide and/or its significant metabolites.

(c) *Data requirements for new registration.* The following data are required to evaluate the effects that a pesticide may have on the environment, particularly on fish and wildlife resources. All toxicity studies should investigate effects of several dosages or concentrations, ranging from an effect level to a nondiscernible-effect level. Proper statistical design and evaluation of any toxicity study is required. For acute and subacute toxicity tests, the results should be stated in terms of an LD–50 or LC–50. When the LD–50 or LC–50 is reported, it must be accompanied with calculations of the slope of the dose-response line, together with 95 percent confidence intervals for the LD–50 or LC–50 and the slope. The data provided will be used in all computations which determine whether the pesticide is subject to a rebuttal presumption against registration or is presumptively classified for restricted or general use. In addition to the quantified indices of lethality, other results, such as symptoms produced, also should be reported.

(1) *Basic tests*—(i) *Avian Acute Oral LD–50.* The single dose oral LD–50 of the technical grade material is required for 1 species of wild waterfowl, preferably the Mallard, or one of the following species of upland game bird: Bobwhite or other native quail, or the ring-necked pheasant.

(ii) *Avian Subacute Dietary LC–50.* The subacute dietary LC–50, 8-day protocol (5-day dietary exposure), of the technical grade material is required for 1 species of wild waterfowl and 1 species of upland game bird. Preferred species are those specified in paragraph (c)(1)(i) of this section. One of the 2 species selected for these studies must be the same species selected for the acute oral study required in paragraph (c)(1)(i) of this section.

(iii) *Fish Acute Toxicity.* Acute toxicity data (LC–50, 96-hr protocol) using the technical grade material are required for a cold-water and a warm-water fish. Similar data may also be required for certain formulated products depending on use patterns and other factors. The rainbow trout and the bluegill are preferred.

(iv) *Invertebrate Acute Toxicity.* Acute toxicity data (LC–50, 96-hr protocol) similar to that in paragraph C are required for a sensitive aquatic invertebrate, such as *Daphnia* sp.

(2) *Conditional tests*—(i) *Acute and subacute tests.* (A) Mammalian toxicity data as required for evaluating hazard to humans and domestic animals will normally be adequate to indicate hazard to wild mammals. Data on toxicity to livestock will be useful, if available.

(B) Acute toxicity data (LC–50, 96-hr protocol) using either the technical grade material, the formulated product, or both are required for shrimp and crabs if the pesticide will be used in, or may contaminate, estuarine or marine environments.

(C) Acute toxicity data for oyster larvae (LC–50, 96-hr protocol) or shell deposition data for a representative marine mollusc, such as the American oyster, are required, using either the technical grade material, the formulated product, or both, if the pesticide will be used in, or may contaminate, estuarine or marine environments.

(D) Satisfactory evidence is required that, under conditions of proposed use, the pesticide or specified formulations will not cause unreasonable adverse effects on the flora and fauna in aquatic environments, including those of the water column and bottom sediments. Because of the complexity of aquatic ecosystems, specific requirements will be established on a case-by-case basis. Toxicity and residue studies may be required using a typical bottom-feeding species (channel catfish or carp); predator fishes representative of cold and warm water habitats (bass, bluegill, northern pike or walleye, and brook or rainbow trout); molluscs (oysters or freshwater clams); Crustaceans (Daphnia, Gammarus, or crayfish); and insect larvae (midges) or nymphs (mayfly).

(ii) *Chronic Tests.* (A) Reproduction studies on Bobwhite and Mallard normally will be required for materials which are persistent, are stored, or accumulate in plant or animal tissues, or are used under conditions where wild birds may be subjected to repeated or continued exposure.

(B) Subacute or chronic fish and/or invertebrate reproduction studies normally will be required if the pesticide is to be used in, or is likely to contaminate, aquatic environments. Judgement on the

necessity for these studies will take into consideration the expected persistence of the pesticide in soil and the aquatic environment, its propensity for bioaccumulation, and use patterns.

(iii) *Special Studies.* (A) Potential hazards relating to a proposed pattern of use may require special testing under actual or simulated field conditions.

(B) Toxicity data beyond those outlined here may be requested on an individual basis and will be specific to the problem.

(C) See Petitions for Tolerances for the data requirements to establish a tolerance or an exemption from the requirement for a tolerance, where a pesticide use may result in residues in fish or shellfish.

(d) *Data requirements for reregistration.* The minimum data requirements for reregistration and classification of outdoor pesticide uses include the avian subacute dietary LC–50 and the 96–hr LC–50 for fish and other aquatic organisms. If these data have been submitted previously, they need not be developed again. Additional studies may be required if a registered pesticide use has demonstrated hazard to fish and/or wildlife—for example, any verified accidental kill of nontarget organisms.

PETITIONS FOR TOLERANCES

§§ 162.83–162.93 [Reserved]

PACKAGING, STORAGE AND DISPOSAL

§§ 162.94–162.96 [Reserved]

GUIDELINES FOR REGISTERING PESTICIDES IN THE UNITED STATES

APPENDIX

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PART I. SCOPE AND INTENT OF APPENDIX

Although, because of bulk, the Appendix to the Guidelines for Registering Pestcides in the United States has been compiled separately from the guidelines, the two are complementary documents. For an integral treatment of a topic, the user should consult both documents: the guidelines primarily explain what the procedural and data requirements for registration are; the appendix delineates how these requirements may be acceptably satisfied.

To facilitate reference between corresponding parts and topics, the appendix is divided into the same major parts as the guidelines. Because many subjects are treated more extensively in the appendix, some of the parts are divided into subparts. Parts presently designated "reserved" will, as the appendix is progressively developed, comprise procedural exhibits, such as data submission forms, and other material that may assist a registrant in compiling data or in completing accurate application packages.

This initial publication of the appendix describes those test methods, procedures, or protocols related to developing registration data for: (1) Product efficacy; (2) environmental chemistry; and (3) hazards to humans and domestic animals and fish and wildlife. Because of the diversity of the materials, the information is presented in several formats:

Bibliographic citations only, where material cited is available in open literature and the method or protocol is acceptable precisely as presented.

Annotated bibliographic citations, where a modification, adaptation, or explanatory statement is necessary for acceptable data development for registration.

Full text (with source credit) of unpublished methods or protocols or those that are not readily available.

Full text of procedures compiled from several sources, authorities, or references.

The appendix is not intended to set standard test methods or prescribe inflexible registration requirements. Accordingly, methods, procedures, and protocols described or referenced are those presently considered adequate for developing data acceptable for registration purposes, or those that can be identified as sources of information within the present state of the art. The Agency recognizes that applicants may be aware of other test procedures which are equally effective for particular purposes and that new procedures will be developed in the future. It also recognizes that the test methods described sometimes may not be well suited to the evaluation of particular products. Thus, modification of the specific procedures set out in the appendix is permissible, providing the intent and reliability of the suggested methods are met. In cases where an applicant proposes to deviate from the procedures suggested, prior agreement with the Agency on the specific data development methods to be employed is strongly recommended.

Neither the guidelines nor the appendix are static documents. They will be expanded and revised periodically to reflect new scientific knowledge, new trends in pesticide development, and new Agency policies. All applicants, and new applicants in particular, are encouraged to consult with the Office of Pesticide Programs to resolve any questions relating to data requirements or data development. Any applicant who considers that certain provisions of the guidelines or the appendix may not be appropriate for a particular pesticide should submit a written statement of his position and consult with the Office to determine what data are necessary in his particular case.

In the course of preparing this appendix, the Agency has actively sought the advice of such distinguished professional groups as the American Institute of Biological Sciences, the Association of Official Analytical Chemists, the Chemical Specialties Manufacturers Association, and the American Society for Testing and Materials.

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PART IV—PRODUCT PERFORMANCE

SUBPART A—ANTIMICROBIAL AGENTS

This section considers antimicrobial agents, primarily efficacy data requirements for supporting labeling claims, and areas in which products are used. The applicant should assure himself that the test method selected to substantiate efficacy is current and applicable to the product proposed for registration.

Efficacy data requirements for registration are designed to provide:

1. Some evidence that the applicant can reproduce the product formula so that each production batch will have the same level of activity.

2. Evidence that the formula which is being offered for sale will have reasonable shelflife.

3. A sufficient volume of testing so that product deficiencies which may be subsequently found on enforcement samples in

[1] More detailed Contents pages precede each part or subpart.

laboratories cannot be ascribed to variations in testing methodology that may be utilized among different laboratories.

Reference to "batches" in this subpart means individually formulated preparations of the product, either in the laboratory or from production.

STERILIZERS

Horowitz, William, ed. 1970. Sporicidal test—official final action. Pages 64–65 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Since "sterilizer" is an absolute term (to destroy all living things), the data required to support a sterilizing claim are greater than those required to support a sporicidal claim. The test requires 30 replicates on each of 2 kinds of carriers (porcelain penicylinders and surgical silk suture loops) against spores of both *Bacillus subtilis* and *Clostridium sporogenes* on 3 different batches (total of 120 carriers on each batch). Thirty carriers of each type must also be tested against spores of *B. subtilis* and *C. sporogenes* on duplicate samples of 1 batch aged to represent a 60-day shelflife stability study (total of 240 carriers, plus 360 carriers equals 600 carriers). No growth must be observed in any of the subculture or resubculture tubes from the 600 carriers.

[illegible]

Horowitz, William, ed. 1970. Sporicidal test—official final action. Pages 64–65 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

The AOAC Sporicidal Test requires 30 replicates on each of 2 kinds of carriers (porcelain penicylinders and surgical silk suture loops) against spores of both *Bacillus subtilis* and *Clostridium sporogenes* on 3 different batches (total of 120 carriers on each batch). Thirty carriers of each type must also be tested against spores of either microorganism on duplicate samples of 1 of the 3 batches, aged to represent a 60-day shelflife stability (total of 120 carriers for the stability test). Added claims for effectiveness against sporeforming organisms other than *B. subtilis* and *C. sporogenes* must be substantiated with data derived from 30 replicates (porcelain penicylinders) on 1 batch. The spores must be inactivated in 30 out of 30 trials at the dilution and exposure time specified. For a statistical confidence level of 95 percent, killing in at least 59 out of 60 trials is required.

VIRUCIDES

(Tentative method prepared by Registration Division, Office of Pesticide Programs, EPA, 1972)

Because no specific official standardized method for evaluating the virucidal activity of disinfectants has been adopted, EPA will accept adequate data developed by any virological technique which is recognized as technically sound. The procedure employed must simulate actual in-use conditions. For this reason, acceptable virological data are usually developed by carrier methods which are modifications of either the AOAC Use-Dilution Method (for liquid surface disinfectants) or the AOAC Germicidal Spray Test (for spray surface disinfectants). To simulate in-use conditions, the specific virus to be tested must be inoculated onto hard surfaces, allowed to dry, and then treated with the product according to the directions for use on the product label. The treated surfaces must be assayed after a 10-min exposure to determine if the virus has been inactivated. Since most germicidal chemicals are inherently toxic for the host systems employed in virological techniques (especially cell cultures), either a "detoxification" procedure, which will render the treated virus free of residual germicide, or a virus concentration of sufficient titer to demonstrate at least a 3-log reduction, even when cytotoxicity is observed in tissue culture systems, must be employed in the study.

The following information must be provided on each test:

1. The dilutions employed in the recovery of the virus film from the surface; e.g., 10^{-1} 10^{-2}, etc.

2. Virus controls: surfaces contaminated with the test virus; no germicide used; virus recovered at various dilutions in assay system (tissue culture, embryonated egg, mouse injection, etc.).

3. The activity of the germicide on a minimum of 5 separate surfaces (e.g., glass slides) contaminated with the test virus, showing recovery at various dilutions in assay system.

4. Cytotoxicity controls: germicide on surfaces without test virus; effect on assay system.

5. The ID–50 values calculated in the various tests.

6. The reduction of the virus titer by the activity of the germicide ID–50/ml of the virus control less the ID–50/ml of the test system, expressed as Log_{10} and calculated by a statistical method (Reed and Muench, 1938; Litchfield and Wilcoxan, 1949, as examples).

App. IV. A–4

A typical laboratory report of a single test with 1 virus involving a tissue culture, therefore, would include the details of the methods employed together with a table such as the following:

Dilution of virus from surface	Virus-germicide (recovery [1] of virus from treated surface)	Virus-control recovery [1] of virus from untreated surface)	Cytotoxicity-control (Toxic effect of germicide; no virus)
10^{-1}	T T T T T	+ + + + +	T T T T T
10^{-2}	T T T T T	+ + + + +	T T T T T
10^{-3}	T T T T T	+ + + + +	T T T T T
10^{-4}	T T T T T	+ + + + +	T T T T T
10^{-5}	T T T 0 0	+ + + + +	T T T T T
10^{-6}	0 0 0 0 0	+ + + + +	0 0 0 0 0
10^{-7}	0 0 0 0 0	+ + + + +	0 0 0 0 0
10^{-8}	0 0 0 0 0	+ + + + +	0 0 0 0 0
10^{-9}	0 0 0 0 0	+ 0 0 0 0	0 0 0 0 0

[1] Demonstrated by cytopathogenic effect, fluorescent antibody, plaque count, animal response, or other recognized acceptable technique.

NOTE.—T=toxic; +=virus recovered; 0=no virus recovered.

Claims of virucidal activity for a product must be restricted to those viruses which have actually been tested. Duplicate studies on one sample of the product are required for each virus tested. When cytotoxicity is evident (as in above table) at least a 3-log reduction in titer must be demonstrated. The virucide must demonstrate complete inactivation of the virus at all dilutions.

Where the test virus does not demonstrate measurable cytopathogenic reactions in subculture growth systems, *in vitro* testing would be considered presumptive. In this case, confirmatory testing *in vivo* must be carried out in a susceptible host after exposure of the virus to the disinfectant.

References

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *Jour. Pharma. Exp. Therapy*, 96: 99–115.

Reed, L. J., and H. Muench. 1938. A simple method of estimating 50 percent endpoints. *Amer. Jour. Hygiene*. 27: 493–497.

DISINFECTANTS—HOSPITALS AND RELATED INSTITUTIONS (LIQUID PRODUCTS)

Horowitz, William, ed. 1970. Use-dilution method—official final action. Pages 61–64 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

With products recommended for disinfection in hospitals, clinics, dental offices, etc., 60 replicates are required on each of 3 samples, representing 3 different batches, against Staphyloccus aureus. Thirty replicates on each of these 3 samples are also required against Salmonella choleraesuis and Pseudomonas aeruginosa. One of these batches, aged to represent a 60-day shelflife stability study, must also be tested with 30 replicates on duplicate samples against S. aureus. The product must be effective in disinfecting 59 out of each set of 60 replicates to provide a 95 percent confidence level of effectiveness as required by EPA. However, where only 30 replicates are required, the product must be effective in disinfecting each set of 30 replicates.

DISINFECTANTS—HOSPITALS AND RELATED INSTITUTIONS (SPRAY PRODUCTS)

Horowitz, William, ed. 1970. Germicidal spray products—official final action. Pages C8–71 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

With products recommended for disinfection in hospitals, clinics, dental offices, etc., 60 replicates are required on each of 3 samples, representing 3 different batches, against *Staphylococcus aureus*. Thirty replicates on each of these 3 samples are also required against *Salmonella choleraesuis* and *Pseudomonas aeruginosa*. One of these batches, aged to represent a 60-day shelf life stability study, must also be tested with 30 replicates on duplicate samples against *S. aureus*. The product must be effective in disinfecting 59 out of each set of 60 replicates to provide a 95 percent confidence level of effectiveness as required by EPA. However, where only 30 replicates are required, the product must be effective in disinfecting each set of 30 replicates.

DISINFECTANTS—HOUSEHOLD AND NONMEDICAL INSTITUTIONS (LIQUID PRODUCTS)

Horowitz, William, ed. 1970. Use-dilution method—official final action. Pages 61–64 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

EPA requires 60 replicates on each of 3 samples, representing 3 different batches, against *Salmonella choleraesuis*. In addition, 30 replicates on each of these 3 samples against *Staphylococcus aureus* and 1 other pathogen exclusive of *S. aureus, Salmonella typhosa* and *S. choleraesuis* must be tested. Data must be developed to show that the other pathogen tested will survive the carrier drying step utilized in the referenced tests. One of these batches, aged to represent a 60-day shelf-life stability study, must also be tested with 30 replicates on duplicate samples against *S. choleraesuis*. The test methods and confidence level requirements are the same as those described for hospitals and related institutions.

Some manufacturers make disinfectant claims for their abrasive kitchen cleaning products. When a disinfectant claim is made, the same data as above is required when the product is tested at 1 part of product to 2 parts of water. In this test, a slurry results; it is allowed to settle, and the test is conducted on the supernatant.

DISINFECTANTS—HOUSEHOLD AND NONMEDICAL INSTITUTIONS (SPRAY PRODUCTS)

Horowitz, William, ed. 1970. Germicidal spray products—official final action. Pages 68–71 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

EPA requires 60 replicates on each of 3 samples, representing 3 different batches, against *Salmonella choleraesuis*. In addition, 30 replicates on each of these 3 samples against *Staphylococcus aureus* and 1 other pathogen exclusive of *S. aureus, Salmonella typhosa* and *S. choleraesuis* must be tested. Data must be developed to show that the other pathogen tested will survive the carrier drying step of the referenced test. One of these batches, aged to represent a 60-day shelflife stability study, must also be tested with 30 replicates on duplicate samples against *S. choleraesuis*. The test methods and confidence level requirements are the same as those described for hospitals and related institutions.

MINIMAL DISINFECTANT CLAIMS (LIQUID PRODUCTS)

Horowitz, William, ed. 1970. Use-dilution method—official final action. Pages 61–64 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Minimal disinfectant claims are limited to situations where it is primarily important to achieve effectiveness against Gram negative enteric bacteria. These claims are allowed on products that are recommended for the disinfection of toilet bowls and urinals. Required are 60 replicates on each of 3 samples, representing 3 different batches against *Salmonella choleraesuis*. One of these batches, aged to represent a 60-day shelflife stability study, must also be tested with 30 replicates on duplicate samples against *S. choleraesuis*. The confidence level requirements are the same as those described for institutional disinfectants.

MINIMAL DISINFECTANT CLAIMS (SPRAY PRODUCTS)

Horowitz, William, ed. 1970. Germicidal spray products—official final action. Pages 68–71 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Minimal disinfectants claims are limited to situations where it is primarily important to achieve effectiveness against Gram negative enteric bacteria. These claims are allowed on products that are recommended for the disinfection of toilet bowls and urinals. Required are 60 replicates on each of 3 samples, representing 3 different batches against *Salmonella choleraesuis*. One of these batches, aged to represent a 60-day shelflife stability study, must also be tested with 30 replicates on duplicate samples against *S. choleraesuis*. The confidence level requirements are the same as those described for institutional disinfectants.

DISINFECTANT CLAIMS AGAINST OTHER MICROORGANISMS (LIQUID PRODUCTS)

Horowitz, William, ed. 1970. Use-dilution—official final action. Pages 61–64 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Offical Analytical Chemists, Washington, D.C.

Claims for effectiveness against specific microorganisms other than those named in the AOAC Use-Dilution Method can be accepted only when supported by efficacy data derived from 10 replicates on 1 batch against each microorganism claimed. The product must be effective in disinfecting each set of 10 replicates. Data must be developed to show that the other microorganisms tested will survive the carrier drying step utilized in the referenced tests.

DISINFECTANT CLAIMS AGAINST OTHER MICROORGANISMS (SPRAY PRODUCTS)

Horowitz, William, ed. 1970. Germicidal spray products—official final action. Pages 68–71 in Official Methods of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Claims for effectiveness against specific microorganisms other than those named in AOAC Germicidal Spray Products Method can be accepted only when supported by efficacy data derived from 10 replicates on 1 batch against each microorganism claimed. The product must be effective in disinfecting each set of 10 replicates. Data must be developed to show that the other microorganisms tested will survive the carrier drying step utilized in the referenced test.

FUNGICIDES (PATHOGENIC TO MAN AND ANIMALS)

Horowitz, William, ed. 1970. Fungicidal tests—official final action. Pages 65–66 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Claims against specific pathogenic fungi must be supported with efficacy data derived from duplicate samples of 1 batch using the AOAC Fungicidal Test. The highest dilution that kills the fungal spores within 10 min is considered the highest dilution that could be expected to disinfect inanimate surfaces contaminated with pathogenic fungi.

SANITIZING RINSES (HALOGENS)

Horowitz, William, ed. 1970. Available chlorine germicidal equivalent concentration—official final action. Pages 63–64 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Sanitizing rinse claims for iodophors, mixed halides and chlorine-bearing chemicals must be substantiated with efficacy data derived by the Available Chlorine Germicidal Equivalent Concentration Method. Data from 1 test on each of 3 samples representing 3 different batches, showing the concentrations of the product equivalent in activity to the reference standards of 50, 100 and 200 ppm of available chlorine as sodium hypochlorite, against *Salmonella typhosa* are required. Data from duplicate samples of 1 of these batches, representing a 60-day shelflife stability study, showing concentrations of the product equivalent in activity to the 3 reference standards must be furnished. Chemical analyses may be substituted for biological testing on the 60-day sample.

SANITIZING RINSES—FOOD CONTACT SURFACES (APPLICABLE CHLORINE GERMICIDAL EQUIVALENT CONCENTRATION NOT AVAILABLE)

Horowitz, William, ed. 1970. Germicidal and detergent sanitizers—official final action. Pages 66–68 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Sanitizing rinse claims for quaternary ammonium compounds, chlorinated trisodium phosphate and anionic detergent-acid formulations must be substantiated with efficacy data derived by the AOAC Germicidal and Detergent Sanitizers Method. Data from 1 test on each of 3 different batches against both Escherichia coli and Staphylococcus aureus are required, along with data from duplicate samples of 1 of these batches representing a 60-day shelflife stability study against E. coli. When claims for the effectiveness of the product in hard water are made, data derived from tests on duplicate samples of 1 batch against both E. coli and S. aureus are required at the hard water tolerance claimed. The efficacy data must show a 99.999 percent reduction in the count of number of organisms within 30 sec for a product to be acceptable as a sanitizing rinse.

SANITIZING RINSES—NONFOOD CONTACT SURFACES (PREPARED BY REGISTRATION DIVISION, OFFICE OF PESTICIDE PROGRAMS, EPA, 1972)

To substantiate the sanitizing claims for a product, the applicant must submit data prior to registration to show that the product, when used as directed, will substantially reduce the numbers of test microorganisms on a treated surface over that on an untreated control surface. The following protocol may be utilized:

Product samples representing 3 different preparations must be tested against each test bacterium on each representative surface depending on the uses proposed on the label. In addition, 1 sample preparation representing a 60-day shelflife stability study must be tested in duplicate using 1 test bacterium. The test microorganisms may be *Staphylococcus aureus* ATCC 6538 and *Klebsiella pneumoniae*, aberrant, ATCC 4352; *Enterobacter aerogenes* ATCC 13048 may be substituted for *K. pneumoniae*. Representative test surfaces may include, but are not limited to, glass, metal, unglazed or glazed ceramic tile, or vitreous china. The propagation of cultures

and the use of subculture media and other related equipment should be as specified in sec. 4.001 and 4.002 of the current AOAC Manual of Methods, 1970.

Determine the count of bacteria in an 18- to 24-hr broth culture and add a 0.01 to 0.03 ml quantity of the broth culture by spreading on 1 x 1 in. sq of test surface using a bacteriological loop. The squares should be dried for 20-to-30 min in a bacteriological incubator at 30 to 37°C. A "zero time" bacterial numbers recovery test must be performed to show the efficiency of the recovery process and must be reported. The "zero time" test should show the loss in viability that occurred during the drying. Apply the product to the test surface as directed on the label. Run parallel tests on the formulation with active ingredient(s) omitted in an identical manner to serve as the controls. After a suitable time interval, recover test organisms by washing the squares with adequate agitation in appropriate media or diluting fluid containing appropriate neutralizers. Make plate counts on appropriate nutrient agar containing the same neutralizers by the pour or spread plate technique. Exposure time intervals between zero time and 10 min should be tested for the product as well as for the untreated controls.

The results must show a bacterial reduction of at least 99.9 percent over the parallel control count within 5 min. Three replicates of each test surface with each test microorganism must be tested against each sample for a total of 9 determinations with each bacterium. The 60-day shelflife study should be carried out with 2 replicates of each test surface against 1 test bacterium.

Reference

Horowitz, William, ed. 1970. Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

RUG AND CARPET SANITIZERS OF BACTERIOSTATS (TENTATIVE METHOD PREPARED BY REGISTRATION DIVISION, OFFICE OF PESTICIDE PROGRAMS, EPA, 1971)

Equipment—1. *Carpet mounting board.* Mount a piece of ¼-in. tempered hardboard, with tempered surface up, on a 16 x 16-in. base of ¾-in. thick marine plywood, with ¾-in. brads.

2. *Cutting equipment.* 2 x 2-in. sq of ¼-in. acrylic plastic as templates with 3/32-in. holes in the center and 1 sharp knife with a replaceable blade.

3. *Scrub brushes.* 1¼ x 3½-in. surgical hand brush with ⅝-in. nylon bristles.

4. *Extraction bottles.* 8-oz, widemouth, round polypropylene bottles with screw caps (Nalgene 2105 or equivalent) containing 10 stainless steel penicillin cylinders and 100 ml of appropriate neutralizer broth. (Similar style glass bottles may be used, but care must be taken to prevent breakage during shaking.) For phenolic based carpet sanitizers, use AOAC nutrient broth with double strength letheen neutralizer (AOAC letheen broth plus an additional 0.7 g Lecithin and 5 g Tween 80 per liter.)

5. *Spray device.* Adjustable spray atomizer modified to feed from a calibrated test tube or bottle. (A Model 15, DeVilbiss atomizer on a 2-oz bottle graduated with 10 ml marks may be used.)

6. *Carpet.* See "Special Registration Protocol."

Test Cultures and Media—1. *Test bacteria. Staphylococcus aureus,* ATCC 6538, and *Enterobacter aerogenes,* ATCC 13048.

2. *Double strength neutralizer broth.* AOAC letheen broth with an additional 0.7 g Lecithin and 5 g Tween 80 per liter or 0.1 percent sodium thioglycollate and 0.01 percent isooctylphenoxypolyethoxyethanol in pH 7.2 phosphate buffer.

3. *Neutralizer plate count agar.* Standard methods agar (Tryptone Glucose Extract Agar) to which is added 0.7 Lecithin and 5 g Tween 80 per liter.

Bacterial Inoculum. Prepare 8-oz French square culture bottles with nutrient agar b (AOAC, 1970, sec. 4.023(a), (2)). Prepare culture suspensions for inoculation (AOAC, 1970, sec. 4.026). Dilute the standardized suspension in phosphate buffer dilution water to obtain a stock inoculum suspension containing 10 x 10^9 organisms perml (AOAC, 1970, sec. 4.023(f)).

Procedure. 1. Cut the carpet into 8 x 12-in. pieces and, with aid of 2 x 2-in. template, cut 2 rows of 3 squares per row from the backing side of the carpet leaving at least 4 in. between the center of each square. The test squares may be completely cut free, or a preferred method is to leave about ⅛ of an in. of backing intact at each corner of each cut square so that the entire piece of carpeting can be sterilized and inoculated without separation. Mark in the center of each test square with a waterproof marking pen to aid in inoculating the pile surfaces. Cover surface of carpeting with aluminum foil and fold over edges to secure. Steam-sterilize and dry. Foil will prevent contamination of controls during spraying. Only carpet that has been determined to be free of residual antimicrobials on the pile or backing surfaces, following autoclaving by a zone of inhibition overlay technique, should be used.

2. Prepare a standardized stock bacterial suspension by washing the 24 hr growth from nutrient agar b bottles and adjust to a density of 10 x 10^9 organisms per ml with phosphate buffer. Dilute the stock suspension with phosphate buffer containing 0.01 percent isooctylphenoxypolyethoxyethanol to a concentration of 1.0 x 10^7 organisms per ml. Inoculate the previously marked center of each square with 0.1 ml of bacterial suspension. (Retain bacterial suspension for determination of inoculation numbers.) Dry inoculated carpet in a warm incubator at 36° C for 60 min with the foil wrap loosely in place. The scrubbing procedure should be conducted in a biological hood or in a glove box. A simple safety chamber can be constructed from a large plastic bag.

3. Condition brushes by immersing the bristles in separate containers (150-mm glass petri dishes or equivalent) of diluted test solution and a control solution without the active agent for 15 min. (If a control solution is not available, use sterile distilled water containing 0.01 percent isooctylphenoxypolyethoxyethanol.) Fasten 2 pieces of inoculated carpet containing 12 test squares onto a mounting board by nailing each corner with upholsterer's tacks with the foil wrapping positioned so as to protect the controls during the scrubbing treatment. Place board in a safety hood.

4. Apply the portion of the predetermined amount of test solution at a temperature of 25° C uniformly to 1 piece of carpeting containing 6 spots of dried bacterial inoculum. Apply liquids by metered spray. The hand brush dipped into the solution no more than 6 times will deliver about 15 ml of solution. Do not exceed this amount. Spray liquid on rug in the amount over and above 15 ml needed to yield a 99.9 percent reduction or in the amount that equals the recommended liquid application rate per square area of rug. Record the total volume of liquid applied by spray and brush. Solids may be applied by dusting. In this case, record the weight of product applied. Shake excess test solution from conditioned brush and transfer to a dish containing 100 ml of test solution. Dip bristles of brush and transfer amount of retained test solution to a spot on the carpet. Scrub the spot for 30 sec. using 30 circular clockwise strokes and 30 circular counterclockwise strokes. A circular area of pile approximately 3 in. in diameter around each spot should be covered by this treatment. Moderate to heavy pressure should be applied downward on the brush to work the solution to the base of the pile. Repeat dipping of brush into test solution and scrubbing procedure until each of the 6 spots is treated. Allow the treated carpet to remain at room temperature for 60 min. for partial drying of treated areas.

5. While the carpet treated with test solution is drying, spray on an amount of nonactive control solution equivalent to the surface area onto 3 of the spots of dried inoculum on the control carpet. The remaining 3 spots are unscrubbed controls to determine reduction in inoculation numbers due to drying. Care should be taken not to wet or scrub over the unscrubbed control area. Scrub the 3 wet spots in the same manner as test carpet. Allow the scrubbed controls to dry 60 min. at room conditions as for test pieces.

6. Following the 60-min. reaction period, cut each 2 x 2-in. test piece free with flamed forceps and knife. Transfer each 2 x 2-in. piece of carpeting to a separate extraction bottle of neutralizer broth. Shake each extraction bottle vigorously for a minimum of 1 min. to free viable organisms from the carpet fibers. Determine the number of viable organisms in each sample bottle by plating duplicate dilutions in standard methods agar with appropriate neutralizer. Determine the number of viable organisms in 0.1 ml of the bacterial suspension used for inoculating the carpet. Incubate all broth extraction bottles to determine germicidal or incomplete neutralization of the test carpet.

7. Determine the percent reduction of viable organisms by the treatment method by comparing the number of surviving organisms of the treated sample against the average viable count of the scrubbed control sample. A count of 1.0 x 10^8 organisms per ml from the extracted unscrubbed control spots is necessary for a valid test.

Special Registration Protocols. The following protocols must be used to derive data to support the registration of a carpet sanitizer.

1. Three product samples representing 3 separate preparations must be tested against each of the 2 bacteria specified in the method with 2 different types of carpet. A 99.9 percent reduction of test bacteria over the control count must be demonstrated in each case. If the product is intended for use on commercial grade carpeting, 2 representative carpets such as "acrylic" and "polypropylene" tufted-loop type may be tested. No carpeting is available to serve as a standard. If the product is intended for use on wool carpeting, an additional representative sample must be tested. If not, the label must bear a disclaimer for such use. All carpet samples tested must be fully identified, and the pile fiber type, pile yarn weight of finished carpet, pile density and tuft height reported. Adequate controls must demonstrate that bacteriostatic agents in the carpet pile or backing do not interfere with the test results.

2. One sample preparation representing a 60-day shelflife stability study must be tested with 1 carpet sample and 1 test bacterium.

The amount of sanitizer applied to a piece of carpet must be extrapolated to gallons of diluted shampoo to square feet of carpet and this must be declared on the proposed label. In general this would be 12 gal of dilute product to 800 to 1200 sq ft of carpet. If the application is intended for hospitals, a wet vacuum pickup must be specified in the label directions. In no case will the so-called "dry shampoo treatment" be considered for hospital use.

Reference

Horowitz, William, ed. 1970. Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

SANITIZERS—FABRICS AND TEXTILES

Petrocci, A. N., and Paul Clarke. 1969. Proposed test method for antimicrobial additives. J. AOAC 52: 836–842.

For products bearing sanitizing claims, the method of Petrocci and Clarke may be employed to provide efficacy data to support registration, since no official test method is available. The test may be carried out in a washing machine in lieu of the Petrocci and Clarke method. However, if a washing machine is used the general protocols, propagation of cultures, fabric to water ratios, test materials, water temperatures and subculture media must be the same as specified in the Petrocci and Clarke method.

The test organisms are klebsiella pneumoniae, ATCC 4352 and Staphylococcus aureus, ATCC 6538. Tests must be conducted with 3 samples representing 3 product preparations, each sample tested with 3 replicate fabric swatches against the 2 test organisms specified. One sample representing a 60-day shelflife stability study must be tested in duplicate against S. aureus. A 99.9 percent reduction within 10 min must be demonstrated over the control counts.

AIR SANITIZERS AND DEODORIZERS (TENTATIVE METHOD PREPARED BY REGISTRATION DIVISION, OFFICE OF PESTICIDE PROGRAMS, EPA, 1972)

No standard method for evaluating air sanitizer claims has been adopted. Several investigators have shown that the incorporation of glycols (triethylene, propylene or dipropylene glycol) in pressurized formulations at concentrations of 5-to-10 percent significantly reduce airborne bacteria when adequate vapor concentrations are achieved. Air sanitizing products that do not contain such concentrations of glycols must be evaluated by a room or chamber technique. The primary test organism is *Staphylococcus aureus*. Depending on the intended use pattern, other organisms, such as *Salmonella* or *Pseudomonas*, may also be used to provide efficacy data. The results must show a bacterial reduction of at least 99.9 percent over the parallel control count within 5 min.

SELF-SANITIZERS

(Tentative method prepared by Registration Division, Office of Pesticide Programs, EPA, 1972)

To substantiate self-sanitizing claims for a product, the applicant must submit data prior to registration to show that the product when used as directed will substantially reduce the numbers of test microorganisms on treated representative surfaces when compared to the numbers of microorganisms on control surfaces which have been treated with the same product without the active ingredient.

Three product samples representing 3 different preparations must be tested against each test bacterium on at least 5 replicates of each representative surface for which the product is intended for use. In addition, 1 sample preparation representing a 60-day shelflife stability study must be tested in duplicate using 1 test bacterium on at least 5 replicates of each representative surface to be treated. The test microorganisms may be *Staphylococcus aureus*, ATCC 6538, and *Klebsiella pneumoniae*, aberrant, ATCC 4352; *Enterobacter aerogenes*, ATCC 13048, may be substituted for *K. pneumoniae*. Representative test surfaces may include, but are not limited to, glass, metal, unglazed or glazed ceramic title, vitreous china, or wooden flooring depending on the uses proposed on the labels. The propagation of cultures and the use of subculture media and other related equipment should be as specified in sec. 4.001 and 4.002 of the current AOAC Manual of Methods.

Apply the product to the control and test surfaces as directed on the label, allowing for complete drying of the surfaces. Determine the count of bacteria in a 48-hr broth culture and add a 0.01 to 0.03 ml quantity of the broth culture by spreading on 1 x 1 in. sq of each test surface using a bacteriological loop. The squares should be dried for 20-to-30 min in a bacteriological incubator at 30 to 37°C. A "zero time" bacterial numbers recovery test must be performed to show the efficiency of the recovery process and must be reported. The "zero time" test should show the loss in viability that occurred during the drying. A minimum count of 1×10^6 organisms per test surface must be recovered after drying for the test to be considered valid.

After a suitable time interval recover test organisms by washing the squares with adequate agitation in appropriate media or diluting fluid containing appropriate neutralizers. The neutralizers selected must be identified and shown to be effective. Make plate counts on appropriate nutrient agar containing the same neutralizers by the pour or spread plate technique. Exposure time intervals between zero time, 30 min, 1 hr, 3 hr and 6 hr should be tested and reported for each surface treated with the product, as well as for each of the controls.

The results must show a bacterial reduction of at least 99.9 percent over the parallel control count within 3 hr to demonstrate any practical value.

Reference

Horowitz, William, ed. 1970. Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

BACTERIOSTATS—TEXTILE PRESERVATIVES

American Association of Textile Chemists and Colorists. 1968. Antibacterial activity of fabrics, detection of: agar plate method, test method AATCC—90—1965. Pages B–175–B–176 in Technical Manual of the American Association of Textile Chemists and Colorists, vol. 44, Research Triangle Park, N.C.

American Association of Textile Chemists and Colorists. 1968. Antibacterial finishes on fabrics, evaluation of, test method, AATCC 100—1965. Pages B–176–178 in Technical Manual of the American Association of Textile Chemists and Colorists, Research Triangle Park, N.C.

Methods prescribed by the American Association of Textile Colorists and Chemists involving agar plates or liquid culture media may be employed to support residual bacteriostatic claims in the presence of moisture.

RESIDUAL BACTERIOSTATS—LAUNDRY ADDITIVES

Howoritz, William, ed. 1972. Bacteriostatic activity of laundry additives—official first action. Pages 400–403 in 2nd supplement to 11th ed. Official Methods of Analysis of Association of Official Analytical Chemists (Reprinted from *J. AOAC* 55 (2)).

Three samples representing 3 product preparations are tested, with 5, 1-in. sq treated swatches against *Staphylococcus aureus* ATCC 6538 and *Klebsiella pneumoniae*, aberrant, ATCC 4352 on seeded agar for each sample. Duplicate samples of 1 preparation representing 60-day shelflife stability must be tested with 5 replicate fabric swatches on agar seeded with *S. aureus*. Score zone of inhibition along single side of square test fabric samples as 1, so that for 5-replicate plate test, a score of 20 shows that bacteriostasis occurs along all 4 sides of each sample. Effective demonstration of residual bacteriostatic activity requires a zone of inhibition in 18 out of 20 sides.

PHENOL COEFFICIENT

Horowitz, William, ed. 1970. Phenol coefficient—official final action. Pages 59–61 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Any phenol coefficient claim requires testing by the AOAC Phenol Coefficient Method in duplicate on 1 preparation against *Salmonella typhosa*. Where a phenol coefficient is declared against any other bacterium, the test also must be run against each bacterium named.

MYCOBACTERIUM TUBERCULOSIS

Horowitz, William, ed. 1970. Tuberculocidal activity—official final action. Pages 71–72 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Official Analytical Chemists, Washington, D.C.

Claims against *Mycobacterium tuberculosis* must be substantiated with efficacy data derived from the AOAC Tuberculocidal Activity Method on 1 preparation of the product (liquid) under test. If the product is a spray, the procedure must be modified to conform with the AOAC Germicidal Spray Product Method using the media, microorganisms, etc., described in the AOAC Tuberculocidal Activity Method.

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PART IV—PROCUCTION PERFORMANCE

SUBPART B.—AQUATIC PEST CONTROL AGENTS

ANTIMICROBIALS: SWIMMING POOLS AND HUMAN DRINKING WATER SYSTEMS

Horowitz, William, ed. 1970. Water disinfectants for swimming pools—official final action. Pages 68–71 in Official Methods of Analysis of the Association of Official Analytical Chemists, 11th ed. Association of Analytical Chemists, Washington, D.C.

The AOAC Water Disinfectants for Swimming Pools Test is used as the basis for substantiating pool water disinfectant claims and/or claims for the disinfection of drinking water. For each claim, the test is conducted with 3 different batches of the pesticide product against both *Escherichia coli* and *Streptococcus faecalis*. Also, data from duplicate samples of 1 batch of the pesticide product aged to represent 60-day shelf-life stability tested against *E. coli* must be determined.

These data requirements do not apply to simple pesticide formulations of sodium (calcium) hypochlorite, sodium (potassium) dichloro-s-triazinetrione, or trichloro-s-triazinetrione intended for use in swimming

pool water, or to sodium (calcium) hypochlorite in well water, when adequate and appropriate directions for water maintenance are provided on the product label. (The other chlorine-releasing chemicals specified cannot be used for drinking water.) These or other pesticidal chemicals, which are proposed for use in treatments of raw waters that can be expected to contain significant quantities of organic substances or which claim effectiveness against bacteria or other organisms other than those named above, will require testing by procedures other than those set forth above or by appropriate modifications of the AOAC test. Specific modifications will relate to and be contingent on label claims and use patterns.

FISH TOXICANTS, REPELLENTS AND REPRODUCTIVE INHIBITORS

A review of the needs to control fish and the possible physical, biological and chemical controls has been published by Lennon (1970). More specific reviews on fish toxicants and the information necessary for registration of a product have also been published (Lennon et al., 1970 and Schnick, 1972). (See also Part VII. Hazard Evaluation, Subpart B. Aquatic Toxicology.)

References

Lennon, R. E. 1970. Fishes in pest situations. Pages 6–41 in Vertebrate Pests: Problems and Control, Vol. 5. Univ. Calif., Davis.

Lennon, R. E., J. B. Hunn, R. A. Schnick, and R. M. Burress. 1970. Reclamation of Ponds, Lakes, and Streams with Fish Toxicants: A Review. Food Agric. Organ. Fish. Tech. Paper No. 100.

Schnick, R. A. 1972. A Review of Literature on TFM (3-trifluormethyl-4-nitrophenol) as a Lamprey Larvicide. Bur. Sport Fish. and Wildl. Invest. in Fish Control No. 44. 31 pp.

FISH TOXICANTS

Laboratory Studies. In the early testing of a potential piscicide, the toxicity is determined by toxicity test. Accepted guidelines for aquatic toxicity testing are published jointly by the American Public Health Association, the American Water Works Association and the Water Pollution Control Federation (1971). These methods have been carefully thought out, tested and revised frequently over the past 50 yr; deviations from these methods should not be made unless there is considerable reason to do so. Until fairly recently the static toxicity test was the only technique considered, but now flow through toxicity tests are the preferred method. The latter method affords less difficulty in maintaining an adequate supply of dissolved oxygen, permitting only low metabolite accumulation for the test animals, and greater ability to maintain constant toxicant concentrations and constant water temperatures (Sprague, 1969; Sinley, 1973a). Continuous-flow bioassays require more equipment, but trouble-free equipment has been designed (Mount and Brungs, 1967; Sprague, 1969; Sinley, 1973b). A labor-saving jar rinser, jar emptier and automatic liquid measuring vessel have been described for static bioassay (Hesselberg and Burress, 1967).

The referenced methods suggest using the receiving water as the diluent, but in preliminary testing of a piscicide this requirement would not be necessary. However, the researcher should consider the effect of water quality on fish health and on the toxicity of the piscicide being tested so that misleading results will not be obtained (Doudoroff, 1957; Doudoroff and Katz, 1950 and 1953; Hoar and Randall, 1971; Appelgate and King, 1962; Sionim and Sionim, 1973; Murphy, 1970). Factors that should be considered in laboratory testing are listed below.

1. Because temperature will have an important effect, toxicity tests should be conducted at several different temperatures (Macek et al., 1969).

2. All the fish for a given trial should be obtained from the same source because some strains of fish show resistance to various chemicals (Dziak and Plapp, 1973; Ferguson and Bingham, 1966).

3. Although all life stages of the fish should be tested eventually (Olson and Marking, 1973; Garrison, 1968), only fish of nearly equal size should be tested in a given trial (Murphy, 1971).

4. Because mud will alter the toxicity of the compound in field trials (Ferguson et al., 1965), it may be desirable to conduct some tests in containers with varying amounts of mud (Loeb and Stackey, 1966).

5. In addition to testing the toxicity to the target fish, the effect on other vertebrates (Brooks and Price, 1961; MacPhee and Ruelle, 1969b) and on representative invertebrates (Shoettger and Olive, 1961) and plants should also be determined.

6. The rate of deactivation of the piscicide should be determined (Marking and Walker, 1973; Marking and Dawson 1972; Lee et al., 1971; Loeb and Engstrom-Heg, 1971).

Papers dealing with toxicity tests of specific piscicides suggested for review of techniques include Berger et al., 1969; Herr et al., 1967; Haag, 1931; and MacPhee and Ruelle, 1969b.

In acute toxicity test research, the results require special statistical treatment. Probit analysis is commonly used (Finney, 1947; Litchfield and Wilcoxon, 1949), but a discussion of other statistical analyses has been written by Finney (1964). Rachlin and Perlmutter (1968) present an approach for establishing maximum permissible concentrations.

Field Studies. Protocols for field evaluations have been developed by the U.S. Fish and Wildlife Service Fish Control Laboratories (Cumming, 1972). In addition, Lennon and Berger (1970) and Gilderhaus et al. (1969) have summarized field testing conducted to gain registration of a particular piscicide. Equipment for adding chemicals and monitoring the effect has been described by several researchers (Anderson, 1962; Price and Haas, 1963; Ziebell, 1966; Richard, 1962).

A major problem in field applications is the effect different water qualities have on the toxicity. Howell and Marquette (1962) suggest that a test should be conducted on each body of water before adding the toxicant; other researchers have added the toxicant on the basis of laboratory studies and then tried to explain the erratic results (Huish, 1961; Mayhew, 1960; Callahan and Huish, 1968). Other problems in field applications are discussed in publications by Cushing and Olive (1956); Torblaa (1968); Kiser et al. (1963); Hughes and Lee (1973); Koeppe (1961); Bonn and Holbert (1961); and Cohen et al. (1960).

Because such a variety of organisms is involved in field applications, samples may have to be preserved for later analysis at perhaps several laboratories. Cope (1960) suggests methods for collection. Problems of homogenization of samples have been discussed by Benville (1970) and Daniels et al. (1965); problems of extraction of pesticides, by Hesselberg and Johnson (1972), Hughes et al. (1970) and Bevenue and Beckman (1966).

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FISH REPELLANTS

Published literature on fish repellents is difficult to identify, and much of it is of little value because repellency has not been carefully defined, particularly from the standpoint of duration. Summerfelt and Lewis (1967) published a summary on fish repellents. Their paper, along with one by Hansen et al. (1972), describe apparatus for laboratory testing of repellents. Four other papers describe a mixture of laboratory and field testing of repellents to migrating salmon (Brett and MacKinnon, 1954; Alderdice et al., 1954; Idler et al., 1956; Idler et al., 1961), but none of these constitute a protocol for testing repellents. The alternative is to adapt the protocols developed for piscicides.

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FISH REPRODUCTIVE INHIBITORS

No publications pertaining directly to fish reproductive inhibitors have been identified. Basic information on the reproductive physiology of fishes is available in 2 publications (Hoar and Randall, 1969; and Schreck, 1974). Although the research to control sex in fish discussed by Schreck was not very successful, such control would inhibit reproduction.

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Few papers have been identified in the literature concerned specifically with test methods for aquatic herbicides, and most that have appeared deal with greenhouse and laboratory tests rather than field test methods. Since field-testing is the only positive method of showing herbicide efficacy under actual conditions, this section will be concerned only with field test methods. The methods were chosen because they have been used over a period of time, have been generally accepted and have appeared frequently in the literature. Dissipation data are among the most important that must be developed in connection with the use of phytotoxic chemicals in water. Only rarely can the flow

of water be controlled, and the water's ultimate use may be uncertain. Frequently it is used for irrigation, livestock, industrial and domestic water, or as an input into a fishery or recreational impoundment.

Several factors are involved in the dissipation of any material in water. The most important are dilution, sorption, volatility and precipitation by chemical reaction. In the absence of significant volatility, dilution is frequently the factor of greatest importance with several exceptions; the loss of diquat by adsorption from flowing water is one of the best examples. Because of this characteristic, however, diquat is very rarely used in flowing water. Dissipation through dilution is not the loss or disappearance of the chemical, but only a reduction in the concentration. Losses due to biological degradation or photodegradation should be minimal because the periods involved are relatively short.

Simple and direct methods for determining the dissipation of several phytotoxic chemicals in flowing water are published (Bartley, 1967, 1969; Chancellor et al., 1957; Demint et al., 1970; Demint, 1971; Frank et al., 1967, 1970; Nelson et al., 1969) Except for the work reported by Nelson et al. (1969) and Bartley (1967, 1969), little has been done to determine the roles played by factors other than dilution in the disappearance of phytotoxic chemicals in flowing water.

Among the hazards posed by phytotoxic compounds in water are the possible injury to irrigated crops and other desirable plants and illegal residues in food and feed crops. Recent publications by Bruns et al. (1972, 1973) illustrate techniques employed in evaluating the tolerance of crops to chemicals in irrigation water and determining the resulting residues. In addition to limiting application rates and treatment exposures to those actually needed to control algae and/or other aquatic vegetation, other precautions may be necessary. If irrigating crops with water containing recommended levels of phytotoxic chemical results in injury to crop plants or produces objectionable residues in feed or food crops, then precautions must be established to permit acceptable use. These precautions might include use of the water only on certain crops, or irrigating with the water might be delayed until the concentration of the chemical has dissipated to an acceptable level. Similar restrictions may be necessary where livestock, potable or recreational waters are contaminated beyond acceptable levels.

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HERBICIDE USE IN FLOWING WATER

In the past, insufficient attention has been given to characteristics of the water in testing aquatic herbicides. The vast majority of publications, if water quality is mentioned at all, list only a few of the characteristics, such as temperature and pH. A more thorough characterization of the water should be provided. There is no longer any question concerning the effect of water quality on the performance of copper sulfate as an algaecide. And, although little if anything has been published concerning water quality on the efficacy of other phytotoxic chemicals, such as the cocoamine salts of endothall and the esters of 2,4–D, studies with these herbicides have provided ample evidence that water quality is very important. The effects of sediment and other forms of particulate matter in water have been reported (Coats et al., 1966; Cochrane et al., 1967; Nelson et al., 1969; Tucker et al., 1967).

Certain chemical and physical characteristics of flowing water are determined largely by the constant movement and turbulence. Stratification and its attendant effects are rarely observed and discontinuous concentrations of nutrients, oxygen and growth regulating chemicals are of minor importance. Naturally-occurring changes in water quality and nutrient levels, other than dissolved gases, are usually minimal during the residence time of water in a stream or other water conveyance system. In most water conducting systems, the volume of water dealt with over a period of time is large in comparison to the area of soil-water interface and the mass of vegetation supported in the system (Peltier and Welch, 1969). Changes in water quality that do occur are, more often than not, due to inputs of sewage effluents, runoff of rainfall or drainage of excess irrigation water into the waterway.

Flowing water makes the use of chemicals for weed control difficult and costly. Also, any hazards in using a particular chemical may become more critical if flowing water is involved. The phytotoxicity of a compound in water is governed largely by the concentration of the phytocide and the length of exposure or contact between the weeds and the herbicide-bearing water. In some instances, there appears to be a fairly precise relationship between the 2 variables of time and concentration. With acrolein, approximately equivalent results are obtained by either 0.1 ppm applied for 48 hr or 0.6 ppm applied for 8 hr. Because of the requirement for a minimum exposure period, it is necessary to apply a herbicide to all the water flowing past a given point during the entire treatment or exposure time. Most herbicide concentrations must be quite high and, because of the large volume of water treated, the expense is considerable.

Finally, although some type of control or check is always desirable, very often it is impossible to secure these in tests in flowing water and the best alternative is observations made before and after treatment. If the period of time involved is 10 days or less, before and after observations of the same sites are preferable to controls that involve different streams or channels or even widely separated sites in the same system. In flowing water it is sometimes possible to leave an untreated section of a waterway upstream from the site where the treatment is made. These untreated sections provide the most reliable comparisons for determining the efficacy of treatments for control of both algae and other aquatic vegetation.

Algae: Control Methods and Testing Procedures. Algae that present the most common and severe problems in flowing water systems are the attached filamentous forms, such as *Cladophora*. Their effects on the delivery capacity of water conveyance systems is magnified far beyond the total mass of vegetation present. Attached filamentous algae are prevalent in concrete lined canals and flumes, but they may also become attached to soil in some instances. Rocks, logs and rooted submersed or marginal aquatic weeds also are common sites for attachment. The filamentous green algae, because of their size, are usually the most obvious; in cold water from snow melt or high altitude lakes, however, some species of blue-green algae predominate. An attached layer of scum of variable thickness composed of algae, sediment, fungi and many forms of aquatic microfauna usually indicate the presence of blue-green algae.

In addition to attached algae, planktonic forms are usually present in flowing water, but because of their minute size, plankton algae are not often considered a problem. Rarely, macroscopic free-floating algae, such as *Aphanizomenon*, enter canals or streams where they may create havoc in water diverted to irrigation sprinkling systems. Except in very long water distribution systems, such as the California Aqueduct and the Delta Mendota Canal in California, there is little opportunity to control phytoplankton once they have entered canals or streams. Control, if required, should be done in the lakes, reservoirs or other sources of water that are more sedentary and before the water enters streams or other conveyance systems.

Several techniques are recognized for controlling algae in flowing water. Two techniques rely on the direct addition of algaecides to the water (Schachterle, 1962); the additions are commonly referred to as the "slug" method and the "continuous application" method. The "slug" method employs instantaneous or short-term applications of relatively high concentrations of algaecides to restricted volumes of flowing water; "continuous application" implies low concentrations of the algaecides applied over long periods of time and, consequently, treatment of large volumes of water (Bartley, 1967). A third technique involves the use of antifouling, or other, coatings that prevent the attachment of algae either by their toxic properties or by physical characteristics that make attachment difficult (Bartley and Otto, 1964, 1965, 1967). Coatings attempted in the past ranged from copper sheets to paints containing various ingredients. Sites where antifouling materials are most commonly used are flumes constructed of concrete or other material and concrete lined canals.

Slug Method of Treatment. The slug method of applying algaecides in flowing water has been described numerous times (Chancellor et al., 1957; Nelson et al., 1969; Schachterle, 1962), particularly in connection with slug applications of copper sulfate. The method is simple but does require quite precise measurements of the volume of water

flow. The same technique, with little or no modification, is used in slug treatments with herbicides for control of submersed vascular weeds (Agricultural Research Service, 1963; Timmons, 1960).

Successful slug application of algaecides in flowing water is dependent upon: (1) Adequate contact time between the algae and algaecides, and (2) a concentration of the algaecide that will produce a lethal effect on the algae during the contact period provided. From this it is evident that, with some algaecides at least, increasing the contact time will permit the use of lower concentrations; conversely, increasing the concentration will decrease the contact time. Consequently, the contact time is determined by the velocity of water flow and the length of the algaecide-bearing slug of water in the canal or stream. Initially, the length of the slug of treated water designed to provide a 30-min contact period will be twice as long in water flowing at a velocity of 2 ft/sec as in water flowing at 1 ft/sec. The total amount of algaecide applied in the faster flowing water will be twice the amount needed in the water of 1 ft/sec velocity.

The manner of introducing the algaecide into water is not significant, except for crystals or other solids and for volatile chemicals, such as acrolein. Uniform dispersion throughout the body of water does not occur until after the water has travelled some distance (Demint, 1971; Feuerstein and Selleck, 1963). In small (up to 100 cu ft/sec) canals, complete dispersion is usually accomplished during the first 0.25 to 0.5 miles of water flow. This may not hold true for canals carrying several hundred or several thousand cubic feet of water per second.

Quantitative measurements to determine the effectiveness of algaecides on filamentous algae are difficult to obtain. Biomass, percent cover and similar determinations are of value only in theory because variability among checks and controls is normally very great. Consequently, visual observations are usually used to estimate the degree of control. No difficulty is encountered where complete control is accomplished. Where partial control follows the use of an algaecide, however, an estimate of percent control is essential along with some documentation of the change in number of species and their contribution to the mass and total population. Evaluation in terms of "satisfactory" or "acceptable" control are of limited value.

Tests for efficacy should be sufficiently extensive and numerous to establish the most satisfactory concentrations and contact periods, or at least an acceptable range of these. In any one locality or geographic area, 3 tests should be the minimum and 6 or more tests are desirable. The parameters and data determinations that should be recorded and reported are enumerated below.

1. Herbicide application.
a. Length of application (instantaneous slug, for example, dumping from dumptruck; or application extended up to several hours).
b. Formulation or physical state of algaecide (powder, crystalline, liquid, other).
c. Method of application (spray boom, spreader, bag, dumping, other).
d. Application date.
e. Amount applied.
2. Observations on water.
a. Water temperature.
b. Water quality (pH, hardness, alkalinity, conductivity, appropriate anions and cations, turbidity, sediment).
c. Water velocity.
d. Volume of flow.
e. Length of canal or other waterway treated.
f. Type of conveyance (earthen, concrete lined, other).
3. Observations on algae control.
a. Species present before treatment.
b. Amount of algal growth (density, mass, length, other) present before treatment.
c. Species controlled by treatment.
d. Species not controlled.
e. Extent of algae control (miles of waterway).
f. Reduction in total mass of algae.
g. Observation dates.
h. Length of control (period between treatments).
i. Number of treatments needed per season.
4. Control of check treatments.
a. Comparison with untreated section of same waterway.
b. Comparison with conditions before treatment.
c. Comparison of results with those of a similar treatment standard, such as copper sulfate.

Continuous Method of Treatment. The use of this method to control algae in flowing water has so far been limited to the application of copper sulfate (Bartley, 1967). The continuous application method is similar in most respects to the slug treatment; continuous application may be considered as a slug of long or infinite duration. Factors to be considered and data required in respect to the algaecide application are identical. For detailed enumeration of the parameters and data determinations that should be recorded and reported, see discussion on the slug method of treatment.

Observations made on control of algae might differ slightly in that control with the continuous application may not be evident immediately because of the lower dosage one would anticipate employing. If the treatment dosage used is not constant, or if the treatment is of extended duration but discontinuous during the season, then it is necessary to establish the initial and followup treatment rates and the duration of each treatment period in terms of days, weeks or months. The use of checks or controls would also be similar in both slug and continuous application.

The most important differences between the slug and continuous application methods have to do with pollution and residues. Unlike the slug treatment, in which the algaecide concentration at any specific point downstream proceeds from a zero level upward to a maximum and then recedes to zero, the algaecide concentration at a given point downstream from the site of continuous application reaches a maximum level which remains constant until the treatment is discontinued. The effect of dilution on dissipation of the algaecide in the continuous application is transitory and restricted to the beginning and terminal portions of the application.

Because of the possible long-term water contamination, it is necessary (1) that the treatment dosage in continuous applications be sufficiently low to be compatible with the various water uses encountered downstream; (2) that a safe concentration of chemical is achieved through dissipation before use of the water; or (3) that the treatment be interrupted periodically to permit specified use of the water (irrigation of crops for example) from that portion of the channel where dissipation has not progressed to the extent that an acceptable level of algaecide is reached.

Antifouling Coatings. Antifouling coatings have traditionally been used to inhibit the attachment of marine algae and barnacles on ships, pilings and other underwater structures. For many years, the most successful of these have been paints containing some form of copper. More recently, other metals and organic metal compounds, such as tributyl tin oxide, have been incorporated into antifouling paints. Empirical methods for testing other antifouling materials have been tried with varying degrees of success. One has been the use of bituminous, vinyl or other paint bases that, in themselves, are not too effective in preventing the attachment of algae. They do, however, facilitate removal of the attached algae by physical means, such as sweeping or high velocity water jets. The successful use of paint surfaces that continuously slough into the water at slow rate and thereby prevent large-scale attachment of algae has been reported.

Because of the cost, the use of antifouling coatings has been limited to critical surfaces or water delivery systems such as flumes of measuring weirs. But, because of the large increases in the cost of algaecides, such as copper sulfate, and the inability of industry to meet the current needs, strong consideration is being given to greater use of antifouling materials. This is particularly true where the financial return on delivered water, for example, for hydroelectric power generation, has increased sharply.

Efficacy testing of antifouling materials is relatively straightforward, at least in respect to the types and quantities used in the past. Because the materials leach into the water at very slow rates, residues from limited applications should be essentially nil. Large-scale use may change the situation entirely and require an assessment of residues, their levels and dissipation rates and hazards that these might present to crops, humans, livestock and the aquatic environment. Most of the work on developing methods for testing antifouling materials in water distribution systems has been done by the Bureau of Reclamation (Bartley and Otto, 1964, 1965, 1967).

The parameters and data determinations that should be recorded and reported are enumerated below.

1. Herbicide application.
a. Structure and construction material.
b. Surface preparation.
c. Method of application (brush, spray, etc.).
d. Amount applied (thickness, weight, etc.).
e. Curing required.
f. Time of year applied.
g. Formulation used.
h. Amount active ingredient.
i. Size of treated area.
2. Observations on water.
a. Water temperature.
b. Water quality (alkalinity, pH, hardness, appropriate anions and cations, conductivity, turbidity, sediment).
c. Active water delivery period(s).
d. Water volume and velocity.
3. Observations on algae control.
a. Algal species controlled.
b. Algal species not controlled.
c. Date of appearance of algae.
d. Amount of algal growth (density, mass, length, other).
e. Effective life of coating (number of seasons).
f. Period of acceptable control.
g. Observation dates.
4. Control or check treatments.
a. No treatment.
b. Recognized standard antifouling material.

Submersed Vascular Weeds: Control Methods and Test Procedures. Submersed vascular weeds in flowing water, although usually not as numerous in number of species as in static water, create serious problems that often must be attended with minimum delay. Weed infestations that become severe enough to impede the flow of water may cause flood-

ing and, in irrigation systems, lead to inadequate delivery of water to farmers. With multiple-use water, deficiencies may also occur in volumes of water available for generation of electrical power and in supplies of domestic water. Consequently, some method for coping with excessive weed growth in flowing water is essential. No study has been made to determine the social or economic impact of submersed weeds in flowing water; however, some data are available on the extent and cost of weed control in western irrigation systems (Timmons, 1960, 1970).

Almost all of the methods presently used for testing herbicides in flowing water were developed in irrigation canals. This is where water delivery was most critical, where hazards to the aquatic environment were minimal and where the only financial resources were and still are available for such weed control development. Very few attempts are made to control submersed vascular weeds in natural streams; no public or private entity will assume the responsibility for weed control in other than navigable streams.

Up to the present, the "slug" method has been found most practical and best suited for use with the herbicides now available. The earliest chemical treatments for submersed weeds in flowing water were based on this technique (Shaw and Timmons, 1949). The "continuous application" technique is also used to control submersed weeds in irrigation systems. The principal factors limiting greater use are scarcity of herbicide chemicals that perform satisfactorily and cost. A third method that has been tested, but that is used infrequently because of lack of a suitable herbicide, involves treating the soil of weed infested waterways during periods when they do not contain water (Frank, et al., 1963).

Slug Method of Treatment. The discussion of the slug method of treatment to control algae applies to its use to control submersed vascular weeds in flowing water. (For details, see that discussion.)

Continuous Method of Treatment. For detailed discussion, see the section on the continuous application method to control algae in flowing water.

Canal Bottom Treatment. In theory, treatment of the soil during periods when there is no water flow is an ideal procedure for control of submersed weeds in waterways (Frank, et al., 1963). In practice, the method has been erratic, with results ranging from no control to weed eradication lasting several years. The principal hindrance to utilizing this method is the absence of herbicides with characteristics that fix them in the soil and still permit retention in their phytotoxic properties. Most herbicides presently available are inactivated by adsorption in soil, are too rapidly degraded or are subject to varying degrees of leaching depending upon the vagaries of the weather.

Only minimal research has been carried out on the behavior and fate of herbicide residues that occur in flowing water as a consequence of treating the soil in dewatered canals or other temporarily dry waterways (Comes, et al., 1974; Frank, et al., 1967).

If canal bottom treatments are employed, data concerning residues in the water following soil treatment are required. They can be obtained by using a modification of a system of monitoring already published (Frank, et al., 1967 and Frank, et al., 1970). Other parameters and data requirements that should be recorded and reported are listed below.

1. Herbicide application.
a. Formulation of herbicide.
b. Method of application.
c. Treatment rate.
d. Date applied.
e. Incorporated in soil.
2. Observations on water, soil and weather.
a. Type of soil and texture.
b. Type and amount of precipitation and general climatic conditions between application and time water is turned into canal.
c. Size of canal.
d. Water temperature.
e. Water quality.
f. Water velocity and volume.
3. Observations and weed control.
a. Species present before treatment.
b. Amount of weed growth (mass, density, length, other) before treatment.
c. Time of appearance of weeds.
d. Weed species controlled.
e. Weed species not controlled.
f. Reduction in amount of weed growth (mass, density, length, other).
g. Observation dates.
h. Length of control.
i. Frequency of treatments needed for weed control.
4. Control or check treatments.
a. Comparison with untreated sections in same canal.
b. Comparison with weed conditions during previous year(s).
c. Comparison of results with results from other types of treatment with other herbicides.

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HERBICIDE USE IN STATIC WATER—GENERAL CONSIDERATIONS

Chemical and Physical Parameters. For all field tests in static waters, certain chemical and physical parameters should be determined and reported in order to describe the test conditions. These parameters should include, but not necessarily be limited, to the following: pH of water, hardness of water, alkalinity of water, dissolved oxygen in water, temperature of water. The determination of these parameters can be made according to the methods outlined by the American Public Health Association (1965) and Golterman (1971), or by other commonly accepted practices, such as electrometric methods. Other observations, such as the presence of a thermocline or extremely muddy water, should also be reported.

Biological Parameters. Certain biological parameters should be recorded at the beginning of every field test. These should include: plant species present, plant density, stage of growth of target species (seedling, pre-flowering, flowering, senescent, etc.). Other appropriate biological observations should be made as necessary. An example of an additional observation would be the presence of a heavy algae bloom in a test where the target species were vascular plants. Another example would be the presence of a heavy coating of epiphytic algae on the leaves of vascular plants.

Types of Plots. Field tests in static waters may be conducted by treating 4 basic types of plots: (1) Whole ponds, (2) open plots, (3) enclosed plots, and (4) plastic pools or other artificial containers.

Whole pond treatments have been used for many years and most closely approximate actual use conditions for an aquatic herbicide. However, the method has several disadvantages. First, it is difficult, if not impossible, to obtain true statistical replicates. Even adjacent ponds, separated by narrow dikes and located on identical soils, frequently react in dissimilar manners when treated with herbicides. In addition to making replication difficult, this lack of uniform behavior also makes it difficult to compare treated ponds with untreated checks or con-

trol ponds. Usually comparing the same pond before and after treatment is more meaningful than comparing a treated pond with an untreated pond.

Open plots are defined, treated areas in larger bodies of water; the plot is not enclosed or contained by any artificial means. Open plot treatments have been used successfully under a variety of situations. A comparison of open plot treatments with whole pond and plastic enclosure treatments can be found in 3 papers by Walker (1964, 1964, 1965).

There are major differences between whole pond tests and open plot tests. First, is the problem of dilution of the herbicides by the surrounding untreated waters. This makes the size of the plot very important. Although many investigators have used smaller size plots, there is some opinion that, to get meaningful reliable results, open plots should be at least 0.25 acre. With this size plot, border effects can be ignored and data can be taken from the centers of the treated areas. Second, with open plots one must also be cognizant of water movement from currents, tides or even wind action, which might carry the herbicide out of the plot boundaries. One advantage over whole-pond treatments is that, with many plots in one body of water, plot-to-plot variation is less a problem.

Enclosed plots are plots in larger bodies of water which are "sealed off" from the rest of the water mass, usually by plastic film. Enclosed plots have been used by various investigators in the past (Gallagher et al., 1968; Walker, 1964, 1965). They offer the smallest, most reproducible type of plots available for use with naturally-occurring plants, which have not been disturbed or transplanted, and a natural bottom which has been disturbed very little, if at all. They can be quite small because there is no dilution of the herbicide by the surrounding untreated water. This is a distinct advantage over the open plot method. Also, because of their smaller size (5 ft x 5 ft used successfully by Gallagher et al., 1968), visual estimation of herbicidal effectiveness is simpler and probably more accurate. A major concern with enclosed plots is to assure that the plastic is not permeable to the herbicide and that the herbicide is not adsorbed or absorbed by the particular type of plastic being used. It is also important that a tight seal be obtained at the bottom.

Above-ground, plastic-lined swimming pools offer a convenient system for testing potential aquatic herbicides. The method is described in detail by Lawrence and Blackburn (1962). The same care must be taken as with enclosed plots to assure the herbicide is not adsorbed or absorbed by the plastic liner.

The advantage of plastic pools is that a wide number of variables can be studied under semicontrolled conditions and a wide spectrum of weeds can be tested. To find the desired weed species growing under the desired conditions in natural bodies of water suitable for field tests is often an impossible task. On the other hand, the results of plastic pool tests should not be over-extrapolated to natural bodies of water. The substrate added to the pool is "artificial" and may not resemble natural pond or lake sediments in either chemical composition or physical structure. Some degree of testing by either the whole pond or open plot method should be done to confirm the results of plastic pool tests.

ALGAE CONTROL

Plot Types. Algaecides may be tested by 3 of the 4 treatments listed above—entire pond treatment, enclosed plots or plastic pools. Open plots are generally not acceptable for algae control tests because of the drift of algae into and out of the plots over a period of time. If plastic pools are used, soil should be placed in the pool bottom even though the algae may not require it for growth. It should also be determined in advance that the algaecide being tested is not absorbed or adsorbed by the specific type of plastic used for the pool liner.

Application Methods. Application methods may vary with the formulation of the algaecide being tested. The major requirements are (1) that the method used is described in detail and (2) that the method results in reasonably uniform distribution of the algaecide in the test area. The latter is particularly important when treating mats of filamentous algae.

Evaluation of Plots. The types of observations to be made and recorded will depend on the type of algae present. In the case of filamentous algae occurring on the substrate and/or occurring as floating mats, visual estimates of the decline in algae are the only practical method of determining efficacy. In the case of single-celled planktonic forms or small planktonic colonies of cells, more precise methods are available. Direct counts of cells or colonies may be made according to the methods outlined by the American Public Health Association, Inc. (1965). Another method entails measuring chlorophyll in water samples according to any of the methods detailed by Golterman (1971).

Visual estimation of water color and/or turbidity is not normally sufficient for measuring the degree of control of this type of algae when tests are performed in the field.

SUBMERSED VASCULAR PLANT CONTROL

Plot Types. Any of the 4 basic types of plots can be used for attached submersed vascular plants. The choice of plot-type would depend on the objectives of the particular test.

Application Methods. Application methods will vary widely with the formulation of herbicide being tested, the size of the plots and, possibly, the rate of application. It is important that: (1) The method be described in detail; (2) reasonably uniform coverage of the plots be attained; and (3) accurate amounts of herbicide be applied.

Evaluation of Plots. Evaluating the effects of the herbicide on the weed population is somewhat difficult. Although some researchers have attempted to run transects through ponds or to remove and weigh all vegetation from randomly selected areas, etc., the vast majority of tests are evaluated by visually estimating the reduction in vigor and stand density of each weed species present. This is the only really practical means of determining efficacy and is very accurate if done carefully. In deep water, where visibility of the bottom is difficult from a boat or from the shore, SCUBA gear can be utilized to great advantage.

FLOATING VASCULAR PLANT CONTROL

Floating vascular plants fall into 2 categories: attached and free-floating.

Floating — Attached. Floating-attached weeds, such as waterlily and watershield, can be treated very much like submersed species (American Public Health Association, Inc., 1965; Comes and Morrow, 1971; Gallagher et al., 1968). If a liquid spray is being used, complete and uniform coverage of the plot is more important with the floating types because the leaves are receiving the spray directly.

Comes and Morrow (1971) used open plots of 50 ft x 50 ft and 25 ft x 25 ft on waterlily. Longer control was obtained in the 50 ft x 50 ft plots, indicating that plot size can be an important factor if evaluation of results is to extend over a period of time.

Free-floating. Free-floating species, such as duckweed and water-hyacinth, may be tested by the whole-pond, enclosed-plot, or plastic-pool methods discussed. The open plot method is not suitable for free-floating species because the plants will move in and out of the plots from wind action and may even become intermingled with untreated plants.

The method of application is more important with floating plants than with submersed plants. If a spray is used which strikes the exposed leaf surfaces, then uniformity of coverage is just as important as it is when testing herbicides on terrestrial plants. Whatever method of application is used, a detailed description of the method should be included with the results.

Effectiveness of the herbicide can be evaluated by visual estimation of stand density and plant vigor.

EMERSED AND MARGINAL PLANT CONTROL

Emersed and marginal species should be treated in much the same manner as terrestrial weeds. The open-plot method normally would be the only method used, although the other 3 methods might be used under special circumstances. An example of the use of open plots in shallow water is the work of McGilvery and Steenis (1965) on alligatorweed.

As with floating species, the method of herbicide application should be one which insures uniform coverage of the plots, particularly in the case of postemergence treatments.

Plot size has to be fairly large, especially for rhizomatous or stoloniferous species. Plants such as *Phragmites* will rapidly invade a plot from the surrounding untreated areas and impede evaluation of the plot as time progresses. For plants like *Phragmites* the absolute minimum plot size should be one one-hundredth of an acre (21 ft x 21 ft) but larger plots are preferable if they are going to be evaluated for more than 1 yr. Buffer strips or alleys between plots should be at least 6 ft wide to minimize the problem of rhizomes from treated plants in one plot crossing the buffer and sending up shoots in the next plot.

Marginal and emersed plants can sometimes be individually counted, measured, or harvested and weighed to assess the effect of the herbicide, but this is rarely necessary or even justifiable. A careful visual estimation of stand density and plant vigor in relation to control plots is adequate for determining herbicide efficacy.

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SUBPART C

Product performance: Fungicides and nematicides IV.C–1
Recommended practice for field evaluation of fungicides used for controlling celery late blight disease IV.C–1
Recommended practice for field evaluation of fungicides used for controlling rose black spot disease IV.C–6
Recommended practice for field evaluation of fungicides used for controlling rose rust disease IV.C–10
Recommended practice for field evaluation of fungicides used for controlling rose powdery mildew disease IV.C–14
Greenhouse test method for rose powdery mildew fungicides IV.C–18
Test method for evaluating mildewstats on leather IV.C–21
Fabric, cordage and fibers (rot, decay, mold and mildew control) IV.C–24
Method of test for control of fabric mildew by fungistats IV.C–25
Test method for evaluating mildewstats on hard surfaces IV.C–30
Method of test for control of wood mildew by fungistats IV.C–34
Wood preservatives (rot and decay) IV.C–38
Industrial preservatives IV.C–39
Surface unspecified or general IV.C–39
Coatings-interior paints IV.C–39
Paper and paperboard IV.C–40
Use-dilution mildew fungicidal test method IV.C–41

PART IV—PRODUCT PERFORMANCE

SUBPART C—FUNGICIDES AND NEMATICIDES

RECOMMENDED PRACTICE FOR FIELD EVALUATION OF FUNGICIDES USED FOR CONTROLLING CELERY LATE BLIGHT DISEASE

(This procedure, TSD Designation 6.301, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 This method is intended for evaluating the efficacy of fungicides in controlling celery late blight disease under field conditions.

2. Test Site.

2.1 Soil should be suitable for celery growth and climatic conditions favorable for disease development. Overhead irrigation should be available to establish high humidity, where necessary, and disseminate spores.

3. Climatic Conditions.

3.1 Late blight develops best under cool weather conditions. Temperatures favorable for spore germination and infection are 16 to 21° C. Spattering and wind-blown water are the major means of spore dissemination (1, 2).

4. Test Plants.

4.1 Giant Pascal Celery [1] is highly susceptible to *Septoria apiicola Spec.* Other susceptible varieties are acceptable.

[1] Giant Pascal Celery seed may be purchased from W. Atlee Burpee Co., 18th Street and Hunting Avenue, Philadelphia, Pa. 19132.

4.2 Plant seed in 51 by 51 mm plant bands or peat pots in the greenhouse three months before transplanting in the field.

4.3 Transplant seedlings in the field 152 to 203 mm apart within the row.

4.4 Plant rows on 762 mm centers.

NOTE: Superscript arabic numbers refer to information footnotes at the end of the paper. Parenthetical numbers indicate literature citations at the end of the paper.

5. Organism.

5.1 *Septoria apiicola Speg.* is the casual organism of celery late blight disease. *Septoria apiicola Speg.*, published in 1887, has priority over the names *Septoria apii* (Briosi & Cav.) Chester and *Septoria appi-graveolentis* Dorogin and is the correct name (3).

6. Test Plot Management.

6.1 Two growing seasons may be necessary to establish an epiphytotic of celery late blight. No testing can usually be done until the second year. In certain areas where the disease is endemic no establishment may be necessary. In such a case, steps 6.3 and 6.4 may be eliminated.

6.2 Plant celery as in 4.3 and 4.4.

6.3 Obtain infected celery foliage from a diseased field and scatter evenly throughout the plot area.

6.4 Sprinkler irrigate three times a week unless rain occurs with similar frequency. This is most important in spreading the spores of the fungus and producing heavy infection on the young celery plants. The first year infection will generally not be heavy enough or evenly dispersed throughout the plot to permit fungicide testing. However, this may not be true in some areas where natural conditions may hasten spread of infection.

6.5 Celery in plots established in mild climates (e.g., Pacific Northwest, California, Gulf States, Georgia, Florida) will overwinter as viable mature plants. Infections will also overwinter. The second year remove the old plants from every second and third row, leaving the first row of every three rows to serve as a source of inoculum. Replant the second and third rows with seedlings as in 4.3 and 4.4. These replanted seedlings will be used for fungicide testing the second year.

6.6 In areas of the country where winter conditions preclude overwinter celery plant survival, the fungus should be collected on the infected plant material and air dried. This material should be stored in a cool, dry place for future use.

7. Procedure.

7.1 Use a randomized block design.

7.2 Use at least four replications per treatment.

7.2.1 For each replication use 4.6 to 6.1 m of row.

7.2.2 Separate each treatment within rows by 305 to 610 mm buffer zone planted to celery.

7.3 Arrange treatments according to Snedecor's Randomly Assorted Digits Table (4), or a similar random digit table.

7.4 Begin treatments soon after the celery plants are transplanted in the field or according to label directions.

7.5 Apply treatments using one or more of the following:

7.5.1 A row crop sprayer adjusted to deliver the desired rate in gallons per acre or liters per hectare.

7.5.2 A hand sprayer, applying the desired rate in gallons per acre or liters per hectare.

7.5.3 A hand duster [2] applying measured amounts per ft, acre, or hectare.

7.6 Controls.

7.6.1 Use Bordeaux Mixture 4–4–100 (4.5 kg—4.5 kg—379 litre) as the Standard fungicide treatment.

7.6.2 Untreated controls shall be used to determine the disease severity and to provide a basis of comparison of the treatments.

8. Data Determinations.

8.1 Visual estimates of disease incidence or actual counts of random infected leaves should be recorded for each plot. A mean disease incidence for each treatment should be determined and the percent disease control calculated according to the formula below (5, 6):

$$\text{Percent Disease Control (PDC)} = \frac{\text{Disease Incidence in Control (DIC)} - \text{Disease Incidence in Treatment (DIT)}}{\text{Disease Incidence in Control (DIC)}} \times 100$$

8.2 Analyze the variance for all treatments using conventional analysis of variance methods (5) and express significance of results at the 95 percent confidence level.

9. Phytotoxicity.

9.1 The type of phytotoxicity should be reported together with the percent of phytotoxicity. Common types of phytotoxicity are: Leaf burning, chlorosis, and stunting.

10. Reporting.

10.1 The following information should be included in reporting test results:

10.1.1 Product name and EPA Registration Number.

10.1.2 Active ingredient(s) and percentage of each.

10.1.3 Rate of application.

10.1.4 How the product was applied.

10.1.5 Number of plants per treatment, or m (ft) of row.

10.1.6 Number of replications.

10.1.7 Spraying or dusting dates.

10.1.8 Mean percent disease incidence.

10.1.9 Mean percent disease control.

10.1.10 Percent phytotoxicity.

10.1.11 Types of phytotoxicity.

10.1.12 Residue present in marketable portions of plant.

10.1.13 Variety used.

11. Effectiveness.

11.1 Effective products should provide a mean disease control of 70 percent or more.

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RECOMMENDED PRACTICE FOR FIELD EVALUATION OF FUNGICIDES USED FOR CONTROLLING ROSE BLACK SPOT DISEASE

(This procedure, TSD Designation 6.305.1, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

[2] Scott Model A61 hand duster has been found satisfactory for this purpose.

1. Scope.

1.1 This method is intended for field evaluation of the efficacy of fungicides used for the control of rose black spot disease.

2. Test Site.

2.1 Soil suitable for rose growth and climatic conditions favorable for disease development. Overhead irrigation should be available to establish high humidity, where necessary. Black spot disease should be endemic in the test area.

3. Climatic Conditions.

3.1 Spore germination and infection are favored by temperatures of 16 to 21° C. Infection requires a wet leaf surface for a period of 6 to 7 h (1, 2, 3).

4. Test Plants.

4.1 Should be of a variety such as Juliet, susceptible only to black spot disease and highly resistant to other foliar diseases. Other varieties are acceptable.

5. Organism.

5.1 Rose black spot disease is caused by *Diplocarpon rosae* Wolf. Pathogenic races of *Diplocarpon rosae* have been reported as differing in their ability to cause disease in host varieties (4).

6. Procedure.

6.1 Use a randomized block design.

6.2 Use at least four replications per treatment. More replications are needed in areas where the disease incidence is light.

6.3 Each replication should consist of one or more bushes of the suspect.

6.4 Treatments should be arranged according to Snedecor's Randomly Assorted Digits Table (5), or equivalent.

6.5 Treatments should begin before the disease appears, or according to label directions.

6.6 Treatments should be made using one or more of the following depending upon use directions:

6.6.1 A power sprayer adjusted to deliver the desired rate.

6.6.2 A hand sprayer.

6.6.3 A hand duster.

6.6.4 A hose sprayer.

6.6.5 An aerosol or low pressure sprayer.

6.7 Plots should be sprinkler irrigated for 3 to 4 h in the late afternoon, 24 h after treatment. This simulates natural rainfall. If at least 2.5 mm of rain falls, do not irrigate.

7. Controls.

7.1 For standard control spray, use Benlate 50 W 227 g/379 liters (8 oz/100 gal) or Maneb 80W g/379 liters (1½ lb/100 gal), or other known effective chemical, whose identity and rate must be indicated in report.

7.2 Untreated control bushes are used to determine the disease severity and to provide a basis for comparison of the treatments.

8. Data Determination.

8.1 Record disease incidence for each treatment replicate. Use either the visual estimate method of Horsfall and Barrett (6, 7) or determine the percent control on the basis of difference between averages of number of lesions on treated and untreated randomly selected leaves.

8.2 The percent disease control may be calculated as follows:

$$\text{Percent Disease Control (PDC)} = \frac{\text{Disease Incidence in Control (DIC)} - \text{Disease Incidence in Treatment (DIT)}}{\text{Disease Incidence in Control (DIC)}} \times 100$$

9. Effectiveness.

9.1 Effective products should provide a mean disease control of 70 percent or more.

10. Phytotoxicity.

10.1 The type and percentage of phytotoxicity, if any, shall be reported. Common types of phytotoxicity are: Leaf burning, chlorosis, and stunting.

11. Reporting.

11.1 The following information should be included in reporting testing results:

11.1.1 Product name and EPA Registration Number.

11.1.2 Active ingredient(s) and percentage of each.

11.1.3 Application rate.

11.1.4 How the product was applied.

11.1.5 Number of plants per treatment.

11.1.6 Number of replications.

11.1.7 Spraying or dusting dates.

11.1.8 Mean percent disease control.

11.1.9 Mean percent disease incidence.

11.1.10 Percent phytotoxicity.

11.1.11 Types of phytotoxicity.

11.1.12 Light, medium, or heavy residue on foliage.

11.1.13 Color of residue.

11.1.14 Analysis of variance of the data. Express significance of results at the 95 percent confidence level.

Literature Cited

(1) Chester, K. Starr, *Nature and Prevention of Plant Diseases*, The Blakiston Company, Philadelphia, Pa., 1950, p. 138.

(2) Massey, L. M., "Blackspot Can Be Controlled," *American Rose Annual*, AMRAA, Vol 31, 1946, pp. 111–118.

(3) Massey, L. M., "Tests with Fungicides for Blackspot," *American Rose Annual*, AMRAA, Vol 40, 1955, pp. 63–91.

(4) Stewart, R. N., and Semeniuk, P., "Report on Rose Research in the U.S. Department of Agriculture," *American Rose Annual*, AMRAA, Vol 50, 1965, pp. 99–105.

(5) Snedecor, George W., and Cochran, William G., *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, Iowa, 1967.

(6) Horsfall, J. G., and Barratt, J. W., "An Improved Grading System for Measuring Plant Diseases," *Phytopathology*, PHYTA, Vol 35, 1945, p. 655.

(7) Redman, C. E., King, E. P., and Brown, I. E., Jr., *Tables for Converting Barratt and Horsfall Rating Scores to Estimated Mean Percentages*, Eli Lilly and Company, Indianapolis, Indiana, 1962.

RECOMMENDED PRACTICE FOR FIELD EVALUATION OF FUNGICIDES USED FOR CONTROLLING ROSE RUST DISEASE

(This procedure, TSD Designation 6.305.2, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 This procedure is intended for field evaluation of the efficacy of fungicides used for the control of rose rust disease.

2. Tests Site.

2.1 Soil suitable for rose growth and climatic conditions favorable for disease development. Overhead irrigation should be available to establish high humidity, where necessary. Rose rust should be endemic in the test area.

3. Climatic Conditions.

3.1 Spore germination and infection are favored by temperatures of 18 to 24°C. Infection requires a wet-leaf surface for a period of 4 h (1).

4. Test Plants.

4.1 Should be a variety susceptible only to rose rust disease and highly resistant to other foliar diseases. McGredy's Scarlet variety is highly susceptible to rose rust disease.

5. Organism.

5.1 Rose rust disease is caused by *Phragmidium mucronatum* (pers.) Schlecht.

6. Procedure.

6.1 Use a randomized block design.

6.2 Use at least four replications per treatment. More replications are needed in areas where the disease incidence is light.

6.3 Each replication should consist of one to six bushes of McGredy's Scarlet variety.

6.4 Treatments should be arranged according to Snedecor's Randomly Assorted Digits Table (2), or equivalent.

6.5 Treatments should begin before the disease appears, or according to label directions.

6.6 Treatments should be made using one or more of the following, depending upon use directions:

6.6.1 A power sprayer adjusted to deliver the desired rate.

6.6.2 A hand sprayer.

6.6.3 A hand duster.[1]

6.6.4 A hose sprayer.

6.6.5 An aerosol or low pressure sprayer.

6.7 Plots should be sprinkler irrigated for 3 to 4 h in the late afternoon, 24 h after treatment. This simulates natural rainfall. If at least 2.5 mm of rain falls, do not irrigate.

7. Controls.

7.1 For standard fungicide spray, use Maneb 80W 680 g/379 liter (1½ lb/100 gal), or other known effective chemical, whose identity and rate must be indicated in report.

7.2 Untreated control bushes are used to determine the disease severity and to provide a basis for comparison of the treatments.

8. Data Determination.

8.1 Record disease incidence for each treatment replicate. Use either the visual estimate method of Horsfall and Barrett (3, 4) or determine the percent control on the basis of difference between averages of number of lesions on treated and untreated randomly selected leaves.

8.2 The percent disease control may be calculated as follows:

[1] Scott's Model A61 hand duster, among others, has been found satisfactory for this purpose.

$$\text{Percent Disease Control (PDC)} = \frac{\text{Disease Incidence in Control (DIC)} - \text{Disease Incidence in Treatment (DIT)}}{\text{Disease Incidence in Control (DIC)}} \times 100$$

9. Effectiveness.

9.1 Effective products should provide a mean disease control of 65 percent or more.

10. Phytotoxicity.

10.1 The type and percentage of phytotoxicity, if any, shall be reported. Common types of phytotoxicity are: Leaf burning, chlorosis, and stunting.

11. Reporting.

11.1 The following information should be included in reporting testing results:

11.1.1 Product name and EPA Registration Number.

11.1.2 Active ingredient(s) and percentage of each.

11.1.3 Application rate.

11.1.4 How the product was applied.

11.1.5 Number of plants per treatment.

11.1.6 Number of replications.

11.1.7 Spraying or dusting dates.

11.1.8 Mean percent disease control.

11.1.9 Mean percent disease incidence.

11.1.10 Percent phytotoxicity.

11.1.11 Types of phytotoxicity.

11.1.12 Light, medium, or heavy residue on foliage.

11.1.13 Color of residue.

11.1.14 Analysis of variance of the data. Express significance of results at the 95 percent confidence level.

Literature Cited

(1) Cochrane, V. W., "The Common Leaf Rust of Cultivated Rose," *American Rose Annual*, AMRAA, Vol 31, 1946, pp. 131–135.

(2) Snedecor George W., and Cochran, William G., *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, Iowa, , 1967.

(3) Horsfall, J. G., and Barratt, R. W., "An Improved Grading System for Measuring Plant Diseases," *Phytopathology*, PHYTA, Vol 35, 1945, p. 655.

(4) Redman, C. E., King, E. P., and Brown, I. F., Jr., *Tables for Converting Barratt and Horsfall Rating Scores to Estimated Mean Percentages*, Eli Lilly and Company, Indianapolis, Indiana, 1962.

RECOMMENDED PRACTICE FOR FIELD EVALUATION OF FUNGICIDES USED FOR CONTROLLING ROSE POWDERY MILDEW DISEASE

(This procedure, TSD Designation 6.305.3, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 This procedure is intended for field evaluation of the efficacy of fungicides used for the control of rose powdery mildew disease.

2. Test Site.

2.1 Soil suitable for rose growth and climatic conditions favorable for disease development. Overhead irrigation should be available to establish high humidity, where necessary. Rose powdery mildew should be endemic in the test area.

3. Climatic Conditions.

3.1 Spore germination and infection are favored by temperatures of 18 to 24° C and relative humidity of 95 to 99 percent (1, 2, 3).

4. Test Plants.

4.1 The test plants should be of a variety susceptible only to powdery mildew disease and highly resistant to other foliar diseases. Dwarf Crimson Rambler, White Knight, and Command Performance varieties are highly susceptible to powdery mildew. Other highly susceptible varieties are acceptable.

5. Organism.

5.1 Powdery mildew disease is caused by *Sphaerotheca pannosa* (Wallr.) Lev. var. *rosae* Wor.

6. Procedure.

6.1 Use a randomized block design.

6.2 Use at least four replications per treatment. More replications are needed when the disease incidence is light.

6.3 Each replication should consist of one bush of Dwarf Crimson Rambler, and one bush of each other suscept used. The bushes may be planted 0.76 m apart, with 1.8 m buffer zone between replications.

6.4 Treatments should be arranged according to Snedecor's Randomly Assorted Digits Table (4), or equivalent.

6.5 Treatments should begin before the disease appears, or according to label directions.

6.6 Treatments should be made using one or more of the following, depending upon use directions:

6.6.1 A power sprayer adjusted to deliver the desired rate.

6.6.2 A hand sprayer.

6.6.3 A hand duster.[1]

6.6.4 A hose sprayer.

6.6.5 Aerosol or low pressure sprayers.

6.7 Plots should be sprinkler irrigated for 3 to 4 h in the late afternoon, 24 h after treatment, to produce a period of high humidity. If at least 2.5 mm of rain falls, do not irrigate.

7. Controls.

7.1 For standard fungicide spray, use Parnon 15 cc in 3.8 liter of water. Other effective chemicals may be substituted, whose identity and rate must be indicated in report.

7.2 Untreated control bushes are used to determine the disease severity and to provide a basis for comparison of the treatments.

8. Data Determination.

8.1 Record disease incidents for each treatment replicate. Use either the visual estimate method of Horsfall and Barratt (5, 6) or determine the percent control on the basis of difference between averages of number of lesions on treated and untreated randomly selected leaves.

8.2 The percent disease control may be calculated as follows:

$$\text{Percent Disease Control (PDC)} = \frac{\text{Disease Incidence in Control (DIC)} - \text{Disease Incidence in Treatment (DIT)}}{\text{Disease Incidence in Control (DIC)}} \times 100$$

9. Effectiveness.

9.1 Effective products should provide a mean disease control of 70 percent or more.

10. Phytotoxicity.

10.1 The type and percentage of phytotoxicity, if any, shall be reported. Common types of phytotoxicity are: Leaf burning, chlorosis, and stunting.

11. Reporting.

11.1 The following information should be included in reporting testing results:

11.1.1 Product name and EPA Registration Number.

11.1.2 Active ingredient(s) and percentage of each.

11.1.3 Application rate.

11.1.4 How the product was applied.

11.1.5 Number of plants per treatment.

11.1.6 Number of replications.

11.1.7 Spraying or dusting dates.

11.1.8 Mean percent disease incidence.

11.1.9 Mean percent disease control.

11.1.10 Percent phytotoxicity.

11.1.11 Types of phytotoxicity.

11.1.12 Light, medium, or heavy residue on foliage.

11.1.13 Color of residue.

11.1.14 Analysis of variance of the data. Express significance of results at the 95 percent confidence level.

Literature Cited

(1) Coyler, D. L., "Biology and Control of Rose Powdery Mildew," Thesis, University of Wisconsin, 1961, p. 109.

(2) Longree, Karla, "The Effect of Temperature and Relative Humidity on the Powdery Mildew of Roses," New York (Cornell) Agriculture Experiment Station Memorandum, Vol 223, 1939, pp. 1–43.

(3) Massey, L. M., "Understanding Powdery Mildew," *American Rose Annual*, AMRAA, Vol 33, 1948, pp. 137–145.

(4) Snedecor, George W., and Cochran, William G., *Statistical Methods*, 6th Edition, Iowa State University Press, Ames, Iowa, 1967.

(5) Horsfall, J. G., and Barratt, J. W., "An Improved Grading System for Measuring Plant Diseases," *Phytopathology*, PHYTA, Vol 35, 1945, p. 655.

(6) Redman, S. E., King, E. P., and Brown, I. F., Jr. *Tables for Converting Barratt and Horsfall Rating Scores to Estimated Mean Percentages*, Eli Lilly and Company, Indianapolis, Indiana, 1962.

GREENHOUSE TEST METHOD FOR ROSE POWDERY MILDEW FUNGICIDES

(This method, TSD Designation 6.303, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 This method is intended for use in the greenhouse evaluation of foliar-applied fungicides for the control of rose powdery mildew *Sphaerotheca pannosa* (Wallr.) Lev. var. *rosae* Wor.

2. Test Fungus and Rose Plants.

2.1 The causal organism (*S. pannosa*) is an obligate parasite. Cultures can be maintained on susceptible varieties growing in the greenhouse.

2.2 The rose variety used in this method is Dwarf Crimson Rambler which is highly susceptible to infection by *S. pannosa*. It is an easily grown and handled host for culturing *S. pannosa* inoculum in the greenhouse. Other highly susceptible rose varieties may be substituted for Dwarf Crimson Rambler. If a substitution is made the name of the variety used must be stated when test is reported.

2.3 Grow disease free host plants from rooted cuttings in a medium consisting of 1 part sand, 1 part loam, and 2 parts peat moss in 152 mm clay pots. Incorporate ground limestone into soil mix so as to adjust pH to a range of 5.5 to 7.0. Maintain all plants in the greenhouse at 18 to 24°C day- and 13 to 18°C night-temperatures. Maintain plants in a vigorous growing state by adding plant nutrients as needed.

[1] Scott's Model A61 hand duster, among others, has been found satisfactory for this purpose.

[1] Scott's Model A61 hand duster, among others, has been found satisfactory for this purpose.

2.3.1 Regularly water plants by adding water to the medium. Do not splash or wet foliage of plants.

2.3.2 Select disease-free test plants of uniform size and vigor.

3. Treatment, Standard and Control (Check) Replications.

3.1 Replicate each treatment four times and include four replicates of standard fungicide and untreated control plants in each test.

3.2 Arrange plants on greenhouse bench in a randomized block design.

NOTE: Parenthetical arabic numbers indicate literature citations at the end of the paper.

4. Fungicide Application.

4.1 Apply fungicide applications, whether sprays or dusts, according to label directions at weekly intervals for a total of four applications. Test plants are removed from greenhouse and sprayed with a DeVilbiss paint spray gun with a No. 30 head at 172 kPa (25 psi) while the plants rotate on a compound turntable (1). If the plants are dusted a vacuum duster is used (2). Sufficient dust is placed in the duster cup for a good coverage of the plant when using 152 mm Hg of vacuum. Use pressurized spray cans as directed and spray plants while on a compound turntable. After spraying the plants are returned to the greenhouse.

5. Standard Fungicide.

5.1 Use appropriate fungicide as a standard depending on whether a product is to be used as an eradicant or a protectant.

5.1.1 If an eradicant is being tested use Parnon, 1.3 percent, 30 cc in 3.8 liter of water.

5.1.2 If a protectant is being tested use Parnon, 1.3 percent, 15 cc in 3.8 liter of water.

6. Untreated Control.

6.1 Untreated controls (unsprayed or undusted plants) shall be used to determine the disease incidence present.

7. Inoculation.

7.1 Heavily mildewed plants, controls obtained from previous tests, are randomly arranged (3.2) throughout the test plants on the greenhouse bench. The conidia are dispersed daily with 414 to 552 kPa (60 to 80 psi) air jet. Heavy air currents from fans will also discharge conidia and can be used instead of the pressurized air. Disperse spores when spore chains are eight spores or more in length.

8. Rating System.

8.1 Each plant is carefully inspected and rated for powdery mildew incidence one week following the fourth spray application. The percent disease control is determined using the following formula (3, 4):

$$\text{Percent Disease Control (PDC)} = \frac{\text{Disease Incidence in Control (DIC)} - \text{Disease Incidence in Treatment (DIT)}}{\text{Disease Incidence in Control (DIC)}} \times 100$$

8.2 Four single plant replications are rated for each treatment. Average the four readings for each treatment (DIT), the four readings for the control (DIC), and apply these data in the formula given above for PDC.

9. Effectiveness.

9.1 Effective products should provide a mean disease control of 70 percent or more.

10. Phytotoxicity.

10.1 The type and percentage of phytotoxicity, if any, should be recorded when it appears or at the same time mildew ratings are taken.

Literature Cited

(1) McCallan, S.E.A. and Wellman, R.H., "A Greenhouse Method of Evaluating Fungicides by Means of Tomato Foliage Diseases," *Cont. Boyce Thompson Inst.*, CBTIA, Vol. 13, No. 3, 1943, pp. 93–134.

(2) Farrar, M.D., O'Kane, W.C., and Smith, Howard W., "Vacuum Dusting of Insects and Plants," *Journal Econ. Ent.*, JEENA, Vol. 41, 1948, pp. 647–648.

(3) Horsfall, J.G. and Barratt, R.W., "An Improved Grading System for Measuring Plant Diseases," *Phytopathology*, PHYTA, Vol. 35, 1945, p. 655.

(4) Redman, C.E., King, E.P. and Brown, I.F., Jr., *Tables for Converting Barratt and Horsfall Rating Scores to Estimated Mean Percentages*, Eli Lilly and Company, Indianapolis, Indiana, 1962.

TEST METHOD FOR EVALUATING MILDEWSTATS ON LEATHER

(This method, TSD Designation 6.202, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 Low pressure spray bombs and liquid products intended for use to control, prevent or inhibit the growth of fungi which cause mildew on various articles or surfaces must be tested to demonstrate *fungistatic* effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Environmental Pesticide Control Act of 1972. It is designed to determine effectiveness of products (applied according to instructions on the labeling) to control mildew and non-pathogenic fungal growth on articles or surfaces of leather, such as book covers, luggage, shoes, and sporting goods. It also indicates duration of protection afforded, providing a basis for recommending repeat applications. Its use will expedite registration and minimize enforcement action.

2. Summary of Method.

2.1 The test is conducted by treating both sides of 1-in. square sections of vegetable tanned cowhide as specified in label directions for use, allowing the leather sections to dry, then on both sides lightly spraying with an inoculum mixture of *Aspergillus niger* and *Penicillium variable*. The squares are incubated individually for one to four weeks in 453.59 g (16-oz) French square jars containing 25.4 mm (1 in.) of water. Mildew growth on treated and untreated leather surfaces is visually rated weekly.

3. Apparatus.

3.1 Glassware: Two 1000-ml Erlenmeyer flasks or other flasks with cotton plugs suitable for preparing agar and for preparing the diluent. Four 250-ml Erlenmayer flasks with cotton plugs, heat sterilized, for use in preparing suspensions of conidia (two for each fungus). Thirty 453.59 g (16 oz) French square jars (10 for treated replications, 10 for untreated, and 10 for the standard fungicide). Prepare the caps by center drilling and inserting 3.175 mm (⅛ in.) brass bolts approximately 12.7 mm (½ in.) in length for attaching a 63.5 mm (2½ in.) piece of #22 nickel chromium wire, bent to form a hook, to suspend the test samples 38.1 mm (1½ in.) below the jar lid.

3.2 Tissue grinder (Homogenizer): No. 4288B, Arthur H. Thomas.

3.3. Atomizer: DeVilbiss #152 (or equivalent) operated at 68.95 kPa (10 psi).

4. Reagents and Materials.

4.1 Test Organisms.

NOTE: Superscript arabic numbers refer to information footnotes at the end of the paper.

4.1.1 *A. niger* (ATCC 6275).[1] Maintain stock cultures on neopeptone agar (10 g neopeptone, 20 g dextrose, 20 g agar, and 1000 ml water). Incubate new stock cultures 7 to 10 days at 25°C (77°F), then store at 2 to 10°C (35.6 to 50°F).

4.1.2 *P. variable*.[2] Maintained and stored same as *A. niger*.

4.2 Leather: Thirty 25.4-mm (1-in.) squares of vegetable tanned cowhide, 1.0 to 1.5-mm thick. Punch or drill a hole in one corner of each square, 6.35 mm (¼ in.) from corner, to facilitate its suspension on a wire hook.

4.3 Standard Fungicide: 0.5 percent solution of p-nitrophenol in 95 percent ethyl alcohol.

5. Procedure.

5.1 Preparation of Conidial Suspension: Place approximately 12.7 mm (½ in.) neopeptone agar into 250-ml Erlenmeyer flask, plug with cotton, autoclave at 103.42 kPa (15 psi) for 20 min. Seed desired organism (*A. niger* or *P. variable*) at center of each flask and incubate for 7 to 10 days at 25°C (77°F). Prepare diluent containing 0.85 percent sodium chloride and 0.2 percent iso-octylphenoxypolyethoxy-ethanol[3] in distilled water, autoclave at 103.42 kPa (15 psi) for 20 min. Pour approximately 100 ml of cooled sterile diluent over culture surface and shake vigorously to dislodge the spores. Pour this spore suspension into heat-sterilized tissue grinder and reciprocate the piston several times to break up spore chains. Filter suspension through a thin layer of sterile absorbent cotton to remove spore chains and hyphal elements. Conidial suspensions may be stored at 2 to 10°C (35.6 to 50°F) for not longer than four weeks. Standardize test conidial suspensions to contain five million conidia per ml (determine by spore count with hemocytometer counting chamber[4]) by adding sterile diluent.

5.2 Treatment: Place ten leather squares in use-dilution of product for three min. For pressurized products, leather squares are placed on a perforated rubber mat and both sides sprayed with product for specified time at recommended distance. When label directions do not specify duration of spray, squares should be sprayed until wet, being careful to obtain equal wetting on both sides of test specimen. Dip the ten leather squares to be treated with the standard fungicide for three min in p-nitrophenol solution, or spray if using pressurized product (4.3). Ten control leather squares are not treated. Dip them in 95 percent ethanol for three min. All squares remained free from mildew growth. to the next step.

5.3 Place equal volumes of well agitated *A. niger* and *P. variable* spore suspensions (5.1) in a #152 DeVilbiss atomizer (or equivalent), maintain agitation and lightly spray both sides of each leather square with the mixture.

5.4 Incubation: Suspend leather squares in individual 453.59 g (16 oz) French square jars containing approximately 25.4 mm (1 in.) distilled water and incubate at approximately 28°C (82.4°F). Tighten the caps, then back off ⅛ turn to allow for some ventilation.

6. Determination of Results.

6.1 Evaluation: Observations are recorded weekly for four weeks or until abundant growth occurs on the treated squares. Visual observation of presence or absence of mold on leather squares is the criterion for determining the effectiveness of the test product. A low-powered microscope is used in settling doubtful cases.

6.2 Interpretation: For an acceptable product every treated square must be free from fungal growth. The results of this test must be correlated to claims made on the label. The directions for use must specify

retreatment every 7, 14, or 21 days, depending on the length of time all treated test squares remained free from mildew growth. Products which do not permit growth after four weeks of incubation need not specify retreatment schedules other than "as necessary."

Footnotes

[1] Cultures of *A. niger* (ATCC 6275) may be obtained from American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852.

[2] Cultures of *P. variabile* NRRL 3765 (formerly classed as *P. glaucum*) are available from: ARS Culture Collection Investigations Fermentation Laboratory, 1815 No. University St., Peoria, Ill. 61604.

[3] Triton X–100, Rohm & Haas Co., Phila., Pa.

[4] For detailed instructions: Tuite, John, *Plant Pathological Methods Fungi and Bacteria*, Burgess Publishing Co., Minneapolis, Minn., 1969, pp. 183–184.

FABRIC, CORDAGE AND FIBERS (ROT, DECAY, MOLD AND MILDEW CONTROL)

Federal Test Method Standard. December 31, 1968. Mildew resistance of textile materials: soil burial method. Method 5762 *in* Textile test methods No. 191. General Services Administration, Washington, D.C.

This soil burial method may be used to support claims for products intended to control rot and decay and mold and mildew of textiles. A product which passes this test will not require testing by the mixed culture method for use on fabrics that will not contact soil.

Federal Test Method Standard. December 31, 1968. Mildew resistance of textile materials: mixed culture method. Method 5760 *in* Textile test methods No. 191. General Services Administration, Washington, D.C.

This test may be used to support rot and decay and mold and mildew claims when the fabric's use will not contact soil. Products which control growth for a 4-wk incubation may specify retreatment "as necessary."

METHOD OF TEST FOR CONTROL OF FABRIC MILDEW BY FUNGISTATS

(This method, TSD Designation 6.203, was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to control, prevent or inhibit the growth of fungi which cause mildew on various articles or surfaces must be tested to demonstrate *fungistatic* effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide and Rodenticide Act of 1972. It is designed to determine effectiveness of products (applied according to instructions on the labeling) to control mildew and nonpathogenic fungal growth on articles or surfaces composed of *fabric*. It also indicates the duration of protection afforded, thereby providing a basis for recommending when to repeat applications.

2. Summary of Method.

2.1 The candidate fungicide is applied to cotton duck strips according to label directions. The strips are dried and inoculated with a standardized spore suspension of *Aspergillus niger* and *Penicillium variabile*. The strips are then suspended over water in jars and incubated at 28 C. The fungal growth is recorded weekly up to four weeks, depending upon label claims. Any variations from the four week test period are noted on the report. If retreatment is required by the label, the strips are retreated according to label directions and so noted in the report.

3. Test Organisms.

3.1 *A. niger* (ATCC 6275). Maintain stock cultures on neopeptone agar [1] or other suitable media. Incubate new stock cultures seven to ten days at 25 C, then store at 2 to 10 C.

3.2 *P. variabile* (NRRL 3765) [2]. Maintain and store as in 3.1.

4. Test Fabric Preparation.

4.1 Cut 25 by 75 mm strips from 238 to 474 g/m² (10 to 20 oz/yd²) single-filled canvas duck. Fabric that will hang without curling excessively is preferable [3].

4.2 Nutrient additive: The number of invalid tests due to failure of mildew growth on the untreated test fabric can be reduced or eliminated by applying a nutrient solution to the fabric. The following is recommended:

4.2.1 For luxuriant growth: Glycerol nutrient solution:

Percent	
98.725	distilled water
1.0	glycerol
0.1	KH_2PO_4
0.1	NH_4NO_3
0.025	$MgSO_4$ 7 H_2O
0.05	yeast extract (pH 5.3)

Prepare in a flask with a cotton plug and autoclave at 103 kPa for 20 min.

4.2.2 A sufficient quantity of the above solution should be prepared to saturate all the fabric to be used. Soak the fabric in it for three min, or until saturated [4]. The excess liquid should be expressed with the fingers and the strips dried before being inoculated and placed in an incubator. Apply nutrient additive before application of fungicide.

5. Preparation of Conidial Suspension.

5.1 Place approximately 13 mm of neopeptone agar in a 250-ml Erlenmeyer flask, plug with cotton and autoclave at 103 kPa for 20 min. After cooling inoculate with the desired organism (*A. niger* or *P. variabile*) at the center of each flask and incubate for seven to ten days at 25 C. Prepare a diluent which contains 0.85% NaCl and 0.05% Triton X–100 [5] in distilled water. Autoclave at 103 kPa for 20 min. Pour approximately 100 ml of the cooled sterile diluent over the culture surface and shake vigorously to dislodge the spores. Pour this spore suspension into a heat-sterilized tissue grinder and reciprocate the piston several times to break up spore chains. Filter suspension through a thin layer of sterile absorbent cotton to remove spore chains and hyphal elements. Conidial suspensions may be stored at 2 to 10 C for not longer than four weeks. Standardize test conidial suspensions to contain five million conidia per ml (determine by spore counts with a counting chamber [6]) by adding sterile diluent.

6. Apparatus.

6.1 Glassware: Two 1000-ml Erlenmeyer flasks or other flasks with cotton plugs suitable for preparing agar and for preparing the diluent. Four 250-ml Erlenmeyer flasks with cotton plugs, heat-sterilized, for use in preparing suspension of conidia (for two fungi). Thirty 500cc French square jabs (10 for the treated replications, 10 for the untreated and 10 for the standard fungicide). Prepare the caps by center drilling and inserting 3 mm brass bolts approximately 13 mm in length attaching a 50 to 65 mm piece of #22 nickel chromium wire, bent to form a hook, to suspend the test samples in the jar. The hooks should support the fabric samples so that the lower ends are approximately 13 mm above the water.

6.2 Tissue grinder (Homogenizer) No. 4288B Arthur H. Thomas.

6.3 Atomizer, DeVilbiss #152 (or other suitable atomizer) operated at 69 kPa.

7. Standard Fungicide.

7.1 Prepare a solution of 0.3% o-phenylphenol in 95% ethanol.

7.2 Dip ten fabric strips (from 4.) in the 0.3% o-phenylphenol solution for three min, or spray until wet depending on product being tested, then allow to dry before proceeding with inoculation (8.2).

NOTE: Experience has indicated that if cloth lighter than 238 g/m² is used, the concentration of the standard fungicide will have to be increased (0.5% o-phenylphenol in 95% ethanol) to obtain mildew control for four weeks.

8. Procedure.

8.1 Treatment: Ten fabric strips (from 4.) are placed in the use-dilution of the product for three min. With pressurized products, fabric strips are placed on a perforated rubber mat and sprayed according to label directions on both sides with the product. Where label directions do not specify duration of spray, fabric strips should be sprayed until wet, being careful to obtain equal wetting on both sides of the test specimen. This is best done by spraying several light applications alternately to each side of the specimen. Include the ten untreated strips, as controls. All samples are allowed to dry before proceeding with inoculation (8.2).

8.2 Inoculation: Inoculum of *A. niger* and *P. variabile* (5.1) is placed in the DeVilbiss sprayer on a 50:50 basis and thoroughly agitated, then lightly sprayed on both surfaces of the fabric test samples.

8.3 Incubation: The fabric samples are then suspended in individual 500 cc jars containing approximately 25 mm of water, and incubated at approximately 28 C. The caps tightened, then backed off ⅛ turn to allow for some ventilation. Observations are recorded weekly for four weeks, or until abundant growth occurs on treated fabric, or so long as the label claims (7, 14, 21, up to 28 days).

9. Evaluation.

9.1 Visual observations of presence or absence of fungal growth on any of the specimens is the criterion for determining the results of the test. A low-powered microscope is used in determining presence of fungal growth in doubtful cases.

9.2 The results of this test must be correlated to claims to be made on the label or labeling. The directions for use must specify retreatment every 7, 14, or 21 days, as necessary, depending on the length of time that all of the test strips remain free from mildew growth. Products which do not permit growth after four weeks incubation may specify "as necessary".

Footnotes

[1] Neopeptone agar (also known as Sabouraud Agar modified and carried as prepared media by Difco and Baltimore Biological Laboratory) consists of 1% neopeptone, 2% dextrose, 2% agar.

[2] Cultures of *P. variabile* NRLL 3765 (formerly classed as *P. glaucum*) are available from: ARS Culture Collection Investigations Fermentation Laboratory, 1815 North University Street, Peoria, Illinois 61604, or American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

[3] Single-filled B-grade canvas, 238 to 474 g/m² (10 to 20 oz/yd²) can be obtained from Testfabrics Inc., 200 Blackford Avenue, P.O. Box 53, Middlesex, N.J. 08846, and has been found satisfactory for this purpose.

[4] The glycerol acts as a humectant and consequently tends to provide a more equal distribution of moisture to all areas of the substrate. The glycerol, yeast extract and mineral salts also provide the necessary nutrients for fungal growth.

[5] Triton X–100, Rohm & Haas Co., Philadelphia, Pa., or other suitable non-ionic wetting agent such as dioctyl sodium sulfosuccinate, Fischer Scientific Co., as Aerosol OT solid A–349.

[6] For detailed instructions: Tuite, John, *Plant Pathological Methods Fungi and Bacteria*, Burgess Publishing Co., Minneapolis, Minn., 1969, pp. 183–184.

TEST METHOD FOR EVALUATING MILDEWSTATS ON HARD SURFACES

(This method, TSD Designation 6.201, was prepared by Technical Services Division, OPP, EPA, and is being considered by the American Society for Testing and Materials. The ASTM format has been retained.)

1. Scope.

1.1 Products which are intended for use to control, prevent or inhibit the growth of fungi which cause mildew on various articles or surfaces must be tested to demonstrate, *fungistatic* effectiveness. This test is designed to determine effectiveness of products (applied according to instructions on the labeling) to control mildew and non-pathogenic fungal growth on hard surfaces such as painted walls, ceilings, floors, metal, glass, tile, porcelain, and plastic.

2. Summary of Method.

2.1 The test is conducted by taking 6.45 cm^2 (1 in. square) glazed tile sections, previously cleaned and sterilized, immersing them in or spraying them with the candidate fungistatic agent, drying; lightly spraying with an inoculum-food solution, redrying, then incubating under optimum moisture and temperature conditions for one week and rating fungal growth on treated and untreated surfaces.

3. Apparatus.

3.1 Glassware: Two 1000 ml Erlenmeyer flasks or other flasks with cotton plugs which are suitable for preparing agar and for preparing the diluent. The 250 ml Erlenmeyer flasks with cotton plugs, heat-sterilized, for use in preparing suspensions of conidia. Twenty-four sterile petri plates (10 for the treated replications, 10 for the untreated, 4 for oven drying).

3.2 Tissue grinder (Homogenizer): No. 4288B, Arthur H. Thomas C.

3.3 Atomizer: DeVilbiss #152 (or equivalent) operated at 68.95 kPa (10 psi).

3.4 Ceramic tiles: Thirty 6.45 cm^2 (1-in.-square) tiles with glazed upper surface, sterilized for 2 h in hot air oven at 180°C (356°F).

4. Test Fungus and Materials.

4.1 Recommended test organism: *Aspergillus niger* (ATCC 6275). Maintain stock culture on neopeptone agar.[1] Incubate new stock cultures 7 to 10 days at 35°C (77°F), then store at 2 to 10°C (35.6 to 50°F).

4.2 Choice of test fungus may vary depending upon label claims of product being tested. If control of fungi other than *A. Niger* is claimed on product label, use of any other mildew fungus is permissible provided genus and species of test organism are stated. Prepare spore suspensions of all test fungi in the same manner (5.1).

4.3 Agar: Two percent water agar (20 g agar per liter water).

4.4 Sterile Czapek solution: Distilled water 1000 ml; Sodium nitrate 3.g; Potassium dibasic phosphate 1 g; Potassium chloride 0.5 g; Magnesium sulphate 0.5 g; Ferrous sulphate 0.01 g; Sucrose 30.g.

5. Procedure.

5.1 Preparation of Conidial Suspension: Place approximately 12.7 mm (½ in.) neopeptone-dextrose agar into 250 ml Erlenmeyer flask, plug with cotton and autoclave at 103.42 kPa (15 psi) for 20 min. Seed *A. niger* inoculum at the center of the flask and incubate for 7 to 10 days at 25°C (77°F). Prepare a diluent which contains 0.85 percent sodium chloride and 0.2 percent Triton X-100 in distilled water. Autoclave at 103.42 kPa (15 psi) for 20 min. Pour approximately 100 ml of cooled sterile diluent over the culture surface and shake vigorously to dislodge the spores. Pour spore suspension into a tissue grinder which has been heat-sterilized, reciprocate piston several times to break up spore chains. Filter suspension through a thin layer of sterile absorbent cotton to remove spore chains and hyphal elements. Conidial suspensions may be stored at 2 to 10°C (35.6 to 50°F) for not longer than four weeks. Standardize conidial suspensions to contain five million conidia per ml. Determine spore concentration using a counting chamber and adjust to proper concentration with sterile diluent. To prepare test suspension, place 1 ml of the standard conidial suspension in 20 ml sterile Czapek's liquid medium and agitate.

NOTE: Superscript arabic numbers refer to information footnotes at the end of the paper.

5.2 Standard Fungicide.

5.2.1 Prepare a solution of 0.2 percent o-phenylphenol in 95 percent ethanol.

5.2.2 Place ten tiles in the Standard fungicide (5.2.1) for 3 min. Drain off excess liquid. Place five tiles in each of two sterile petri dishes and dry for 40 min. (longer if necessary) at 37°C (98.6°F) with lids ajar.

5.3 Treatment: Place ten tiles in use-dilution of test product for 3 min. Drain off excess liquid. Place five tiles in each of two sterile petri dishes and dry for 40 min. (longer if necessary) at 37°C (98.6°F) with lids ajar. For pressurized products, tiles are placed glazed surface up on a perforated rubber mat and sprayed for specified time at recommended distance. Tiles are then drained, placed in sterile petri dishes and dried as described above.

5.4 Inoculation: After drying, tiles are lightly sprayed on glazed surface with *A. higher*-nutrient suspension as previously described. Avoid overspraying to prevent puddling and washing test product from glazed surface. Tiles are then returned to 37°C (98.6°F) oven and dried with petri dish lids ajar. Ten sterile untreated tiles are inoculated and dried in same manner.

5.5 Incubation: Each tile, sprayed side up, is placed in an individual petri dish containing hardened 2 percent sterile water agar. To avoid large water droplets, usually formed by the heavy condensate on petri dish lids when hot agar is poured, from falling onto the tile surface and diluting the product: (1) Allow the plates to stand and dry for several days before they are used in a test; (2) exchange the wetted lids with sterile dry lids; or (3) seal the tile to the agar surface by placing melted agar around its periphery, then turn the plate upside down. Plates are incubated (preferably in a wet-type incubator) at 25°C (77°F) for seven days.

6. Interpretation of Results.

6.1 Visual observation of presence or absence of mold or fungal growth on tiles is the criterion for determining the effectiveness of the test product. A low-powered microscope is used in settling doubtful cases. Each check tile must be at lease 50 percent covered with fungal growth to consider the test valid. A product is considered to pass the test if no growth appears on any of the treated tiles at the end of seven days incubation. Products qualifying under this test must bear adequate directions for use, including a statement to repeat application when new growth appears, and naming some of the appropriate hard surfaces (such as walls, ceilings, floors, and counter tops) on which it is intended to be used.

Footnotes

[1] Neopeptone agar composed of 1 percent neopeptone, 2 percent dextrose, 2 percent agar.

METHOD OF TEST FOR CONTROL OF WOOD MILDEW BY FUNGISTATS

(This method, TSD Designation 6.204, was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society of Testing and Materials.)

1. Scope.

1.1 Products intended for use to control, prevent or inhibit the growth of fungi which cause mildew on various articles or surfaces must be tested to demonstrate *fungistatic* effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Environmental Pesticide Control Act of 1972. It is designed to determine effectiveness of products (applied according to instructions on the labeling) to control mildew and non-pathogenic fungal growth on articles or surfaces composed of wood which has *not* been painted or coated (a common cause of musty odors in basements and attics). It also indicates the duration of protection afforded, thereby providing a basis for recommending when to repeat applications. Its use will expedite registration and minimize enforcement actions. This test is not satisfactory to support claims to control rot and decay of wood.

2. Summary of Method.

2.1 The candidate fungicide is applied to wood blocks, following label directions. After drying, the blocks are inoculated with a mixed spore suspension of *Aspergillus niger* and *Penicillium variabile*. The blocks are incubated over water in jars at 28 C for four weeks after which the presence or absence of mold growth is recorded.

3. Test Organisms.

3.1 *Aspergillus niger* (ATCC 6275). Maintain stock cultures on neopeptone agar (10 g neopeptone, 20 g dextrose, 20 g agar, and 1000 ml distilled water). Incubate new stock cultures seven to ten days at 25 C, then store at 2 to 10 C.

3.2 *Penicillium variable* (NRRI 3765).[2] Maintain and store as in 31.1.

4.1 Ponderosa pine (*Pinus ponderosa* Laws.) sapwood blocks approximately 2.5 by 2.5 by 1.5 cm. The wood shall be free of excessive resins, knots, growth rings or other abnormalities. Blocks shall be surfaced on four sides and smooth. Wood shall be kiln dried after sawing to avoid infestation of wood rotting fungi and any wood showing evidence of such infestation shall be eliminated as test material. Condition wood at room temperature in a dry room to 15% moisture content before weighing. Calculated weight shall fall between 6.0 and 7.0 g per 16 cm^2 (1 $in.^2$). Blocks containing heartwood areas shall not be used as they will inhibit mold growth under test conditions. Each block is corner drilled to allow hanging of blocks as in 6.1, or other means of hanging can be used.

5. Preparation of Conidial Suspension.

5.1 Pour approximately 50 ml neopeptone agar into 250-ml Erlenmeyer flask, plug with cotton and autoclave at 103 kPa for 20 min. Seed the desired organism (*A. niger* or *P. variabile*) at the center of each flask and incubate for seven to ten days at 25 C. Prepare a diluent which contains 0.85% NaCl and 0.05% dioctyl sodium sulfosuccinate[3] in distilled water. Autoclave at 103 kPa for 20 min. Pour approximately 100 ml of the cooled sterile diluent over the culture surface and shake vigorously to dislodge the spores. Pour this spore suspension into a tissue grinder, heat-sterilized, and reciprocate the piston several times to break up spore chains. Filter suspension through a thin layer of sterile

absorbent cotton to remove spore chains and hyphal elements. Conidial suspensions may be stored at 2 to 10 C for not longer than four weeks. Standardize test conidial suspensions (1) to contain five million conidia per ml (determine by spore counts with a counting chamber) by adding sterile diluent.

6. Apparatus.

6.1 Glassware: Two 1000-ml Erlenmeyer flasks or other flasks with cotton plugs suitable for preparing agar and for preparing the diluent. Four 250-ml Erlenmeyer flasks with cotton plugs, heat-sterilized, for use in preparing suspension of conidia (for two fungi). Thirty 500 cc French square jars (10 for the treated replications, 10 for the untreated, and 10 for the Standard Fungicide). These are prepared by center drilling the caps and inserting 3 mm brass bolts approximately 13 mm long for attaching a 63 mm piece of #22 nickel chromium wire. The wire is bent to form a hook on the end and is used for suspending the test samples in the jar. The length of the hook from the bottom of the cap to the bottom of the hook shall be 51 ± 6 mm.

6.2 Tissue grinder (Homogenizer) No. 4288B Arthur H. Thomas.

6.3 Atomizer, DeVilbiss #152 (or other suitable atomizer) operated at 69 kPa.

7. Standard fungicide.

7. Prepare a solution of 0.5% pentachlorophenol[3] in 95% ethanol.

7.2 Dip ten untreated blocks in the 0.5% pentachlorophenol solution for three min and allow to dry before proceeding with the next step (8.2).

7.3 When testing pressurized products spray Standard fungicide blocks, in a hood, on all sides with the solution (7.1) using an atomizing sprayer and allow to dry before proceeding.

8. Procedure.

8.1 Treatment: Ten untreated blocks are dipped in the use-dilution of the product for three min. With pressurized products, blocks are placed on a perforated rubber mat where they are sprayed, according to label directions, on all sides with the product. Where label directions do not specify duration of spray, blocks should be sprayed until wet, being careful to obtain equal wetting on all sides of the test specimen. This is best done by spraying several light applications alternately to each side of the specimen. Include the ten untreated blocks as controls. All samples are allowed to dry before proceeding to the next step.

8.2 Inoculation: Inoculum of *A. niger* and *P. variabile*, prepared as previously described, is placed in the DeVilbiss sprayer on a 50:50 basis and thoroughly agitated, then sprayed lightly on all surfaces of the test blocks.

8.3 Incubation: The blocks are then hung in individual 500 cc jars containing approximately 25 mm of water, and incubated at approximately 28°C. The caps are tightened, then backed off ⅛ turn to allow for some ventilation. Observations are recorded weekly for four weeks or until abundant growth occurs on treated blocks.

9. Evaluation.

9.1 Visual observation of mold or absence of mold on any of the specimens is the criterion for determining the results of the test. A low-powered microscope is used in settling doubtful cases. The untreated control blocks must have a minimum of 50% fungal coverage to consider the test valid.

9.2 The results of this test must be correlated to claims made on the label or labeling. The directions for use must specify retreatment every 7, 14, or 21 days, as necessary, depending on the length of time that all of the test blocks remain free from mildew growth. Products which permit growth after four weeks incubation may specify retreatment "as necessary".

Footnotes

[1] Cultures of *P. variabile* NRRL 3765 (formerly classed at *P. glaucum*) are available from: ARS Culture Collection Investigations Fermentation Laboratory, Northern Utilization Research and Development Division, 1815 North University Street, Peoria, Illinois 61604, or American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

[2] AEROSOL OT A–349, Fischer Scientific Co., has been found suitable for this purpose.

[3] Baker pentachlorophenol, technical grade, has been found satisfactory for this purpose.

Literature Cited

(1) Tuite, John, *Plant Pathological Methods Fungi and Bacteria*, Burgess Publishing Co., Minneapolis, Minn., 1969, p. 183–184.

WOOD PRESERVATIVES (ROT AND DECAY)

American Wood-Preservers' Association. 1955. Standard method for field tests with stakes. AWPA Method M7–55 *in* AWPA Manual of Recommended Practice.

Separate tests should be conducted for each method of application (pressure, dip, brush, spray, etc.).

American Wood-Preservers' Association. 1956. Standard method for field tests with posts. AWPA Method M8–56 *in* AWPA Manual of Recommended Practice.

Separate tests should be conducted for each method of application (pressure, dip, brush, spray, etc.).

American Society for Testing and Materials. 1974. Standard method of evaluating wood preservatives by field tests with stakes. ASTM Designation: D1758–74.

This method should be used only for pressure methods of application.

INDUSTRIAL PRESERVATIVES

Surface Unspecified or General. American Society for Testing and Materials. 1970. Standard recommended practice for determining resistance of synthetic polymeric materials to fungi. ASTM Designation: G21–70.

This procedure is limited to materials into which a pesticide is incorporated during the manufacturing process.

Coatings-Interior Paints. American Society for Testing and Materials. 1973. Tentative method of test for resistance to growth of mold on the surface of interior coatings in an environmental chamber. ASTM Designation: D3273–73T.

PAPER AND PAPERBOARD

Vinson, L. J. May 1953. The Lever spore cloud method for the evaluation of antimycotic properties of paper and paperboard used in the soap industry. TAPPI 36(5):234–236.

This method may be used for determining the effectiveness of fungicides applied at the dry end of paper and paperboard manufacture. If the paper or paperboard is intended for uses other than the soap industry, the test may be modified by using other organisms.

USE-DILUTION MILDEW FUNGICIDAL TEST METHOD

(This method, TSD Designation 6.206, was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill fungi which cause mold and mildew growths must be tested to demonstrate "cidal" effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Environmental Pesticide Control Act of 1972. It is suitable only to determine effectiveness to *kill* mildew organisms (applied according to label directions). Residual effectiveness, if intended or claimed, must be demonstrated using different tests depending on the nature of the surfaces or articles on which the product is to be used. This method is especially applicable for testing products which are applied after dilution with water. Its use will expedite registration and minimize enforcement actions.

2. Reagents and Materials.

2.1 Purity of water. Unless otherwise specified, reference to water shall be understood to mean distilled water or water of equal purity.

2.2 Culture Medium. Neopeptone-neutralizer broth. Prepare by dissolving 0.7 g lecithin (Asoletcing) and 5.0 g "Tween 80" (sorbitan mono-oleate) in 400 ml of hot water and boiling until dissolved. Add 1.0 g sodium thiosulfate, 10 g neopeptone and 20 g dextrose. Adjust to pH 7.2 ±0.2 using tris buffer (2.2.1), and bring volume to one litre with water and mix thoroughly. Place 10 ml of this medium in cotton-plugged 25 by 150 mm test tubes for sterilization and use. Autoclave at 103 kPa (15 psi) for 20 min.

2.2.1 Tris buffer stock solution: 0.1M. Dissolve 12.1 g tris (hydroxymethyl) aminomethane in 500 ml water and bring to one litre. Approximately 70 ml of this buffer solution is placed in 1 litre of the culture medium, then agitated thoroughly and distributed to test tubes, etc. The pH of the medium will be approximately 7.4 before autoclaving and 7.2 after autoclaving.

2.3 Test Organism. Aspergillus niger (ATCC 6275). Maintain stock cultures on neopeptone agar (10 g neopeptone, 20 g dextrose, 20 g agar, and 1 litre water). Incubate new stock culture 7 to 10 days at 25°C, then store at 2° to 10°C. Conidia of required resistance survive 10-min exposure at 20°C to a phenol dilution of 1:80 (2 parts 5% phenol, 6 parts water), but not to a 1:70 dilution (2 parts 5% phenol, 5 parts water).

2.4 Phenol: Use 5% stock solution and keep in well-stoppered amber bottle, in a cool place, protected from light. [A standard stock solution to be prepared by a qualified chemist: AOAC Manual, 11th Edition. 4.001 (c)].

2.5 Sterile distilled water. Prepare stock supply of distilled water in litre flasks, plug with cotton, and autoclave at 103 kPa for 20 min.

2.6 Asparagine solution: Make stock supply of 0.1% asparagine solution in water in Erlenmeyer flasks of convenient size, plug with cotton, and autoclave at 103 kPa for 20 min. Use to cover metal carriers for sterilization and storage.

2.7 Sodium hydroxide solution: Approximately 1 N (4%). (For cleaning metal carriers before use.)

3. Preparation of Conidial Suspension.

3.1 Place approximately 13 mm (½ in.) of neopeptone agar into a 250 ml Erlenmeyer flask, plug with cotton and autoclave at 103 kPa for 20 min. Seed *A. niger* at the center of the flask and incubate for 7 to 10 days at 25°C.

3.2 Prepare a diluent which contains 0.85% NaCl and 0.2% Triton X–100 in distilled water. Autoclave at 103 kPa for 20 min.

3.3 Pour approximately 100 ml of the cooled sterile diluent over the culture surface and shake vigorously to dislodge the spores. Pour this spore suspension into a tissue grinder, heat-sterilized, and reciprocate the piston several times to break up the spore chains. Filter suspension through one-half layer sterile absorbent cotton to remove spore chains and hyphal elements.

3.4 Conidial suspensions may be stored at 2 to 10°C for not longer than four weeks.

3.5 Standardize test conidia suspensions to contain five million conidia per ml by adding sterile diluent (3.2).

3.6 Determine by spore counts with a counting chamber.[1]

NOTE.—Superscript arabic numbers refer to information footnotes at the end of the paper.

4. Apparatus.

4.1 Glassware: Twenty-four lipless 25 by 150 mm test tubes with cotton plugs. Six petri plates 15 by 100 mm with filter paper. Two 250 ml and one 1000 ml Erlenmeyer flasks with cotton plugs. Sterilize all glassware two h in hot air oven at 180°C.

4.2 Transfer needle: A 5 to 8 cm Pt. alloy wire, No. 18 to 23 B & S gauge. Make 5 mm right angle bend at end of wire and fit other end into suitable holder.

4.3 Racks and Baskets: Any convenient style.

4.4 Water Bath: A suitable water bath for maintaining 20°C for the exposure period of the product.

4.5 Carriers: Polished Cylinders (Penicillin Cups), 8±1 mm o.d., 6±1 mm i.d., length 10±1 mm of type 304 stainless steel[2].

4.6 Tissue ginder: (Homogenizer) No. 4288B, Arthur H. Thomas.

5. Operating Technique.

5.1 Soak metal carriers (4.5) overnight in 1 N NaOH (2.7), rinse with tap water several times, than rinse twice with distilled water; place cleaned carriers in multiples of ten in 25 by 150 mm test tubes with closures (or cotton plugs) and cover with asparagine solution (2.6). Sterilize at 103 kPa for 20 min, cool, and hold at room temperature. When the carriers are cooled, pour off the asparagine and cover with standard spore suspension of *A. niger* (3). and allow to stand 10 min. Pour off the spore suspension and remove the metal carriers and place them (ten each) in a sterile petri plate matted with filter paper. Allow them to dry overnight at room temperature or place them in an oven or incubator at 37°C for 2 h with the lids ajar. Place 10 ml of the use-dilution of the product in each of ten, 25 by 150 mm test tubes with closures. Place 10 mm of sterile distilled water in each of two, 25 by 150 mm test tubes with closure for the viability control (untreated checks). All test tubes are placed in a suitable rack and submerged in a water bath so as to cover the lower 2 in. of the tubes. Allow the tubes to come to temperature equilibrium at 20°C, then place the metal carriers in the test tubes at 30 sec intervals. When the carriers have been in contact with the product for 10 min, remove them with a flamed needle in a manner so as to drain all excess liquid from the carriers and place them individually in 25 by 150 mm test tubes containing 10 ml of neopeptone neutralizer broth (2.2). Immediately after placing carriers in test tubes, swirl tubes 3 times. Incubate all tubes at 25°C for at least three days before evaluating for the presence of fungal growth. Negative readings in ten out of ten replications are considered satisfactory evidence of fungicidal activity of the product. The viability control should read positive in both untreated replications.

6. Interpretation.

6.1 The results of this test must be correlated to claims to be made on the label or labeling. Products which pass this test may be labeled as fungicides or mildewcides which will kill or destroy mold and mildew organisms. However, if the product does not have residual effectiveness, the labeling must state "non-residual" or "destroys on contact only".

Footnotes

[1] For detailed instructions: Tuite, John. 1969. *Plant Pathological Methods Fungi and Bacteria*. pp. 183–184.

[2] Fisher Scientific Company, 7722 Fenton Street, Silver Spring, Maryland 20910.

SUBPART D

Product performance: Terrestrial herbicides and plant growth regulators	IV.D–1
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Testing phases	IV.D–2
Herbicide application	IV.D–3
Other considerations (crops, cultural practices, soil and climatic factors)	IV.D–5
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PART IV.—PRODUCT PERFORMANCE

SUBPART D.—TERRESTRIAL HERBICIDES AND PLANT GROWTH REGULATORS

Pesticides in this group are numerous and extremely diverse in their physical and chemical properties. Moreover, they are applied differently under practical use situations in the field and interact variably with the environment once introduced into the biosphere. These factors directly influence both biological performance (efficacy as a regulator) and residual life (dissipation or persistence).

Test methods for these pesticides also, necessarily, vary widely. For herbicides, currently by far the most important category economically, test methods are almost as numerous as researchers in the area of weed science. For some of the more subtle, less definitive, uses of plant growth regulators, however, there is a paucity of published reports on test methods or protocols. Testing under actual field conditons remains the only acceptable method for evaluating the performance of plant regulators.

Distinct limitations may be noted in this subpart. For example, in the section, "Terrestrial Herbicides," priority is given largely to products that are used selectively in croplands. Herbicidal uses on noncropland and industrial sites are not included. Neither are the uses of herbicides in forests and in specialty home and garden situations discussed. Herbicides for aquatic uses are discussed in Part IV. Subpart B., "Aquatic Pest Control Agents." Desiccants and defoliants are also omitted.

The discussion of plant growth regulators is not fully comprehensive. Attention has been given to a few uses that are representative of products presently being marketed. This category in particular will need to be expanded in future revisions.

TERRESTRIAL HERBICIDES

Methods reported in the literature for testing the effectiveness of herbicides vary with the compound, crop, location, application equipment, type of supplemental cultural practices and/or other factors. In general, multiple test methods are required to prove the effectiveness of a given compound. The methods selected should test the compound in the manner in which it is to be used and in a manner which will show the effect of field variables on the compound's effectiveness. The data obtained should support the product label claims and should be representative of the results that the grower will obtain from commercial usage of the product.

Cropland herbicides may be applied as preplant, preemergence and/or postemergence applications. The terminology used in reference to such applications *in this discussion* of test methods is defined below:

1. *Preplant application*. An application of a herbicide made before planting the crop to control weeds, either before or after the weeds emerge. The interval between the herbicide application and crop planting and the stage of weed growth at the time of application should be identified.

2. *Preemergence application*. An application of a herbicide made after planting and before emergence of the crop to control weeds, either before or after the weeds emerge. The interval between the planting of the crop and the herbicide application and the growth stage of the weeds at the time of application should be identified.

3. *Postemergence application*. An application of a herbicide made after the emergence of the crop to control weeds, either before or after they emerge. The interval between crop emergence and application should be identified in terms of time or crop growth stage. The growth stage of the weeds at the time of application should also be identified.

Testing Phases. Field trials with herbicides to obtain data to show product effectiveness and usefulness and to support label claims can be divided into the 4 test sequences or phases described below:

1. Trials designed to show product effectiveness and to demonstrate the effect of field variables on effectiveness. Trials in this phase should be designed to study those variables encountered under actual commercial use conditions that might influence the product's effectiveness. In this phase, trials may consist of small, replicated plots in 2 or more selected test sites. Trials should be designed to provide data which can be subjected to statistical analysis.

2. Trials designed to confirm product effectiveness and to develop specific label claims. These trials are similar in design to those in phase 1, but the number of test sites are increased to represent more adequately the major production areas of the test crop and the introduced variables are emphasized less. As soon as sufficient data are available to support a specific label claim, the product should be used in accordance with that claim in subsequent trials. When sufficient data are available to support all label claims, except those requiring large-scale trails, this phase is completed.

3. Trials designed to show product usefulness when used in accordance with label claims. This phase includes specific studies to determine the effect of the product upon man, his environment or his food when used according to directions and cautions on the label. A discussion of these studies is covered in Part VI. Environmental Chemistry and Part VII. Hazard Evaluation of this appendix.

4. Trials designed to confirm product effectiveness and usefulness when used in accordance with label claims under actual commercial use conditions. Trials in this phase should be conducted under actual commercial use conditions, that is, with grower equipment, under grower cultural practices and in normal crop production or use areas. Test sites should be representative of each State or geographical area in the major production or use areas and should be representative of the different growing seasons in each area. Plots may or may not be replicated, but they should be large enough to reflect actual commercial use responses; for example, the elimination of small plot effects, the introduction of soil type variables and the introduction of pest incidence variables. This phase may also be used to obtain data to support additional label claims such as aerial and mist blower applications.

The investigator should make a major effort to use methods or procedures in Phases 1 and 2 that will provide data which can be directly correlated with the results obtained in phase 4.

Herbicide Application. Wiese (1972) discusses the importance of precise and uniform application of herbicides, along with the equipment, techniques and procedures required to obtain such application. The prime consideration in the application of herbicides, whether in small plot or large-scale trials, is uniformity of compound distribution and coverage. This is true in preplant, preemergence and postemergence applications.

The application equipment used in testing herbicides in small plots in phase 1 or 2 should be designed to simulate grower equipment. This can be readily accomplished by using the basic components of commercial application equipment in the design of the small-plot equipment. For example, nozzle types, sizes and arrangements can be identical to those used by growers for ground sprayers and should produce comparable results; or single-row commercial granular application equipment mounted on a garden tractor for small-plot trials should produce results comparable to a multiple of such units on a larger tractor. For large-scale trials in phase 4, commercial application equipment should be used. The importance of the number of gallons of diluent used in the application of herbicides depends upon the product and its use. In applications made to the soil before the emergence of the weeds, gallons of diluent used per acre may not be critical. Uniform coverage is the prime consideration and is directly related to the application equipment. In applications made to emerged weeds, where coverage of the foliage is required, the gallons of diluent required depends upon both the application equipment and the density of the weed foliage. For these latter uses, gallons of diluent required should be included as a study item, particularly in phase 1. A gallonage range that is adaptable to the commercial equipment for the particular application should be used.

The quantity of the product used per given area depends largely upon the product and the weeds to be controlled, along with the residual effect desired. Dosage rates should be tested in phase 1 to select an optimum range. In subsequent testing, rates above and below the optimum rate should be tested; the higher rate should indicate the crop safety margin and the lower rate should indicate the minimum effective rate. Test rates in phase 4 should be in accordance with label claims.

Trials should be designed to provide data to support the optimum number and timing of applications for effective control and for crop safety. For preplant applications, the interval between the herbicide application and crop planting should be studied in phase 1 as a variable. For preemergence applications, the interval between the planting of the crop and the herbicide application should be studied. For postemergence applications, the interval between crop emergence and application should be studied in terms of time or crop growth stage.

Appropriate commercial standard herbicides at label rates, along with untreated controls, should be included in all phase 1 and 2 trials. Weedy and/or cultivated checks may be used as the untreated controls. The use of both types of checks is suggested if cultivation is required for supplementary weed control. In large-scale phase 4 trials, the commercial standard in use by the grower should be included. An untreated control is desirable.

OTHER CONSIDERATIONS

Crops. Selected major varieties and types representative of the crop should be included in phase 1 trials. In selecting varieties and types, the investigator should consider those which cover the range of growth characteristics for the crop that might affect the compound's activity; for example, weak versus strong rooted, fast versus slow growing and early versus late maturity. If no varietal response to the compound is shown in these early trials, the varietal choice in subsequent phases is not critical and the investigator can use those varieties most adaptable to his test site. If a varietal response is noted in phase 1 trials, subsequent trials should include varieties and types as a variable.

The planting rate should be within the range recommended for the crop in the particular test area. Row spacings common to the crop in the test area should normally be used. For some crops, it may be desirable to include the extremes in recommended row spacings to study shading effects. A range of planting depths within the range recommended for the crop should be included in phase 1 studies with preplant and preemergence applications. Data obtained from these trials should reflect any effects of varying planting depths on the incidence of crop injury that might be encountered under commercial use conditions. In subsequent trials, commercial planting equipment at recommended depth settings should be used. If planting depth is found to be a critical variable, crop emergence and crop injury ratings should be taken from all trials.

Cultural Practices. Cultural practices for a given crop vary from production area to production area and frequently from grower to grower within an area. The effects of cultural practices on the product's effectiveness, therefore, are important.

Selected methods of seed-bred preparation common to the test crop should be studied. In phase 1 trials, it is suggested that such methods cover the extremes from poorly to well prepared seed-beds to study effects of clods and plant debris on product effectiveness. These extremes should be within the limits normally encountered by growers. If seed-bed preparation does not affect weed control in these early trials, the seed-bed preparation in subsequent trials is not critical.

Cultivation practices should be those common for the crop in the test site. Since many crops are being grown under minimum tillage practices, it may be desirable in phase 1 trials to determine the effectiveness, both initial and residual, of the product without cultivation. If cultivation is required to supplement the product's effectiveness, subsequent trials in phase 2 should be designed to show the effects of the product used without cultivation, the effects of cultivation only and their combined effects. Some cultivation practices may reduce the effectiveness of the product through movement of untreated soil; these should be recognized and studied as variables. Trials in phase 4 will be influenced by the investigators' choice in grower cooperators. Cooperators should be selected who are knowledgable in the crops, pests and land management practices reflected in the label claims to be supported.

Irrigation practices should be studied as a variable if the product is to be used in irrigated area. The influence of different irrigation practices must be studied in the use area and is best studied, therefore, in phase 2 or 4.

Normally, fertilization practices are not a consideration in testing herbicides and should be within the range recommended for the crop in the test site.

Soil and Climatic Factors. Selected soil types available within test sites in phase 1 should be included in trials with preplant, preemergence and postemergence applications, especially where the product is used primarily for preemergence weed control. In subsequent trials, in phases 2 and 4, soil types should be considered in choosing test sites. Variations in soil types at a given site also should be a consideration in phase 4, and rating of weed control and crop safety should reflect the effects of these variations.

Records of adverse climatic conditions and the influence of rainfall and temperature on the effectiveness of the product should be maintained in all trials. Test sites chosen for phases 2 and 4 should be representative of the use areas and use seasons and should provide the data required to show the relationship between rainfall and/or temperature and product effectiveness.

Measuring Plant Responses. Frans (1972) discusses plot designs and field evaluation procedures along with statistical treatment. For registration purposes, adequate counts and/or ratings should be taken to show the effectiveness of the compound against individual weed species and to show crop safety. For preplant and preemergence applications, data should be taken on crop emergence, stand, crop injury and weed control. Periodic ratings should be taken to reflect time and percent emergence. The data on crop injury and weed control should show initial and final responses, with sufficient ratings in between to show progression or regression of symptoms. Similar data on crop injury and weed control should be taken for postemergence applications. Whenever possible, the crop injury or weed control data should be interpreted in terms of "acceptability;" this can be done by reference to the commercial standard or to the untreated control, or by reference to accepted rating systems, such as yield and quality (grades) of the produce.

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PLANT GROWTH REGULATORS

Many bioassay, laboratory and greenhouse methods have been published for the detection of chemicals which modify plant growth. Only a few published methods are available, however, for evaluating the potential commercial utility of these chemicals under field conditions.

Testing under actual field conditions remains the only acceptable method for evaluating the efficacy of potential plant regulators. Because responses obtained with plant regulators cover such a wide range, for example, bud inhibition, berry size increase, fruit coloring, specific field test methods are outlined. These are based to a large extent on methods employed to evaluate the efficacy of growth regulators presently marketed. New

methods and protocols for evaluating plant growth regulators are continually being developed because both new uses and new chemicals are being sought. In all cases, however, data obtained from field tests must support the label claims for the product and must be representative of the results that the producer can expect when the product is used in accordance with label instructions under commercial conditions.

CONTROL OF AXILLARY BUD (SUCKER) GROWTH ON TOBACCO

Plant growth regulating chemicals have been developed which prevent the growth of suckers. These growth inhibiting chemicals may be divided into 2 types: Systemic and contact. The systemic regulators are applied as sprays to leaves, are absorbed by the plant and are translocated throughout the plant, but especially to the meristematic areas of the axillary buds. The contact type sucker control chemicals are also applied as sprays but are directed down the stalk so that they contact the axillary buds. They kill the small suckers without damaging more mature leaf tissue. Test methods and factors affecting the efficacy of various tobacco sucker control agents have been discussed in a number of publications (Collins et al., 1972; Seltmann and Priest, 1972; Seltmann and Marshall, 1967; Smith et al., 1971; Steffens et al., 1969a; Steffens et al., 1969b; and Spaulding et al., 1970).

The Regional Tobacco Growth Regulator Committee, composed of State and Federal researchers, extension workers, representatives of tobacco and chemical industries and representatives of the Environmental Protection Agency, have worked together to evaluate new plant growth regulating chemicals potentially useful in tobacco production. Two types of field tests are conducted annually, usually at 15 locations in 10 States. Tobacco is evaluated with respect to efficacy of treatment, agronomic characteristics, commercial useability, chemical and physical properties and smoke taste. Samples of known history are made available for assessment of residues of applied chemicals.

The flow chart (Figure 1) summarizes the progression of tests established. After each test, the Committee will evaluate the date to determine if any entry is eligible for the next test in the progression. After company proprietary tests, the Committee makes recommendations to the Environmental Protection Agency regarding label registration.

APPENDIX TO GUIDELINES FOR REGISTERING PESTICIDES

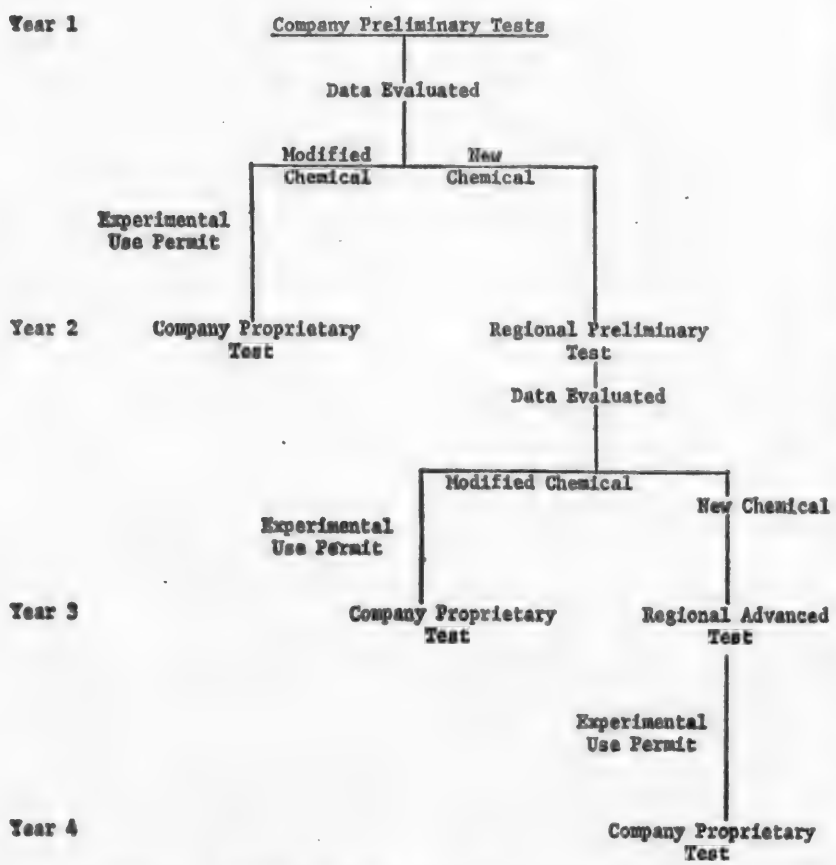

Figure 1. Testing Schematic for Tobacco Sucker Control.

TEST CHEMICALS

New Chemicals. These include structures, mixtures or sequential treatments of chemicals not previously tested. Two or more chemicals to be treated in sequential applications will be considered "one" treatment. If sequential treatments involve 2 new chemicals or one new chemical and one which has already been passed by the Committee, the treatment will be considered a new chemical.

Modified Chemicals. These include formulations of previously approved chemicals which have been slightly altered (salt form, surfactant, etc.). The company submitting the chemical must supply data to indicate if the entry is to be considered a modified chemical.

TYPES OF TESTS AND TEST METHODS

There are 4 sequential levels of testing: company preliminary tests; regional preliminary tests; regional advanced tests; and company proprietary tests.

Company Preliminary Tests. These tests are field trials conducted by chemical companies to compile data to justify entry of a chemical into the regional testing program. The test requirements include:

1. Minimum of 2 flue-cured and 2 burley tobacco locations, if the chemical is to be entered in both flue-cured and air-cured tobacco classes. Both locations for any 1 class are to be in the area where the class is normally produced.
2. 15-plant plots (nonreplicated).
3. Efficacy based on sucker weight and number; the test chemical must be 90 percent as effective as the performance of the appropriate standards included in the same test. (See Test Standards.) Data are to be obtained for flue-cured tobacco 2 wks after treatment (observational) and at final harvest (sucker number and green weight). For burley tobacco, sucker number and green weight are to be taken at harvest.
4. Phytotoxicity data taken at the appropriate stage (scale 0 to 5); leaf drop is to be noted.
5. Records of cultural practices and environmental conditions.

Regional Preliminary Tests. These tests are conducted by State or Federal agricultural experiment station scientists who are Committee members. General testing requirements are:

1. The formulation of the chemical (or closely related formulation) must have been tested on field-grown tobacco.
2. Each person conducting the test must know the chemical composition of the active ingredient on a confidential basis.
3. The company is to supply all information available on toxicity to humans, animals, etc. and any information available on the breakdown, persistence, action, etc. of the chemical.
4. The company is to supply sufficient quantities of the formulated chemical to conduct the tests at all proposed locations. The formulated chemical is to be packaged in containers which can be shipped to the individual locations by the specific dates stipulated by the Committee.
5. The company submitting the chemical is to suggest a rate and is to specify whether the chemical will be used alone or as part of a sequential treatment. The company also is to indicate if it is to be considered a new or modified chemical.
6. After application of the formulation, and during the test, the research worker will determine, by visual observation, the potential of the chemical for achieving the desired results. This information will be forwarded immediately to the coordinator for that tobacco class so that information on efficacy across a particular class can be summarized. After a decision is made by the

coordinator, based on the information available, complete data will be collected from only those treatments showing the desired results.

Field test procedures and data requirements for regional preliminary tests include the following:

1. Suggested distribution for flue-cured tobacco, 6 locations; for burley tobacco, 4 locations; for cigar filler tobacco, 2 locations; for dark tobacco, 2 locations; and for Maryland tobacco, 1 location.

2. 30-plant plots (nonreplicated).

3. Agronomic observations.

a. Efficacy based on percent sucker control by weight.

b. Phytotoxicity data taken after 2 days and at a maximum manifestation (0 to 5 scale). Leaf drop is to be noted.

c. Yield.

4. Cured leaf data (on successful entries only).

a. Quality evaluations based on percent usable by tobacco companies.

b. Chemical and physical properties (composite sample by class). (See Table 1.)

c. Smoke-taste tests to include major off-flavor screening only. (Tobacco companies conducting smoke panel tests must know identity of applied chemicals.)

d. Residue determinations reported to the Committee for information purposes only.

Requirements for passing the regional preliminary test are listed below. (See also Test Standards.)

1. Minimum sucker control based on sucker weight must be 90 percent as effective as the performance of the appropriate standards included in the same test.

2. Leaf injury must average 2.5 (0 to 5 scale) or less with no leaf drop and must be compared to appropriate standards.

3. Warehouse evaluation, physical and chemical properties determinations and smoke tests results must compare favorably with appropriate standards.

Regional Advanced Tests. After successfully passing the preliminary tests, chemicals may be entered in advanced tests, also conducted by State and Federal scientists-Committee members. General requirements include:

1. The identity of the active ingredient of an entry must be revealed as public information. The identity of the complete formulation of an entry must be revealed to the overall test coordinator on a confidential basis.

2. The company is to supply all information available on toxicity to humans, animals, etc. and any information available on the breakdown, persistence, action, etc. of the chemical.

TABLE 1—REQUIRED CHEMICAL AND PHYSICAL PROPERTY ANALYSES

Flue-cured tobacco	*Burley tobacco*
Nicotine or total alkaloids.	Nicotine or total alkaloids.
Reducing sugars	Total N.
Total volatile bases	Alkalinity number of water-soluble ash.
Total volatile bases minus nicotine.	Equilibrium moisture content.
Total ash	Filling value.
Equilibrium moisture content.	Total volatile bases.
Filling value	Total volatile bases minus nicotine.
	Total ash.

3. The company is to supply sufficient quantities of the formulated chemical to conduct the tests at all locations proposed. The formulated chemical is to be packaged in containers which can be shipped by the company to the individual locations by the specific dates stipulated by the Committee.

4. Only 1 application dosage per chemical treatment (based on data obtained from the regional preliminary test) will be tested in a given regional advanced test for any 1 tobacco class.

Field test procedures and data requirements for regional advanced tests include the following:

1. Suggested distribution for flue-cured tobacco, 6 locations; for burley tobacco, 4 locations; for cigar filler tobacco, 2 locations; for dark tobacco, 2 locations; and for Maryland tobacco, 1 location.

2. Plot size—randomized block design with 4 replicates, 30 plants/replicate. (See Test Standards.)

3. Agronomic data.

a. Efficacy based on percent sucker control, sucker number, and sucker weights for full season.

b. Phytotoxicity data taken after 2 days and at maximum manifestation (0 to 5 scale).

c. Cured leaf yield.

4. Cured leaf data.

a. Warehouse evaluation based on usability and body and texture for flue-cured tobacco; usability and quality rating for burley tobacco.

b. Chemical and physical property determinations. (See Table 1.)

c. Smoke-taste tests (off-flavors). The company is to state if the taste is objectionable.

5. Residue determinations.

a. Sampling procedure. Cured leaf will be taken from 3 stalk positions: bottom third, middle third, and top third. Whole leaf samples will be submitted by location to the chemical company; midrib tissue will be included in the sample analyzed.

b. Sample size. A 25-leaf sample will be drawn at random for each of the stalk positions mentioned above. If large plots are involved, several samples will be taken and subdivided. Residues will be determined on flue-cure and burley tobacco and possibly also on the other tobacco classes involved in the regional tests.

c. Analytical methods. The chemical company will develop reliable analytical methods for residue determinations and submit the residue data to the Regional Committee, if permissible. Upon request, analytical methods will be made available by the company to individual members of the Regional Committee on a confidential basis.

d. Specialized residue analysis may be conducted on cigarettes manufactured from treated tobacco.

Requirements for passing the regional advanced test are listed below. (See also Test Standards.)

1. Minimum degree of sucker control must be 90 percent as effective as appropriate standards.

2. Leaf injury must average 2.5 (0 to 5 scale) or less with no leaf drop due to chemical.

3. For flue-cured tobacco, the specified chemical and physical properties should fall between those of the standard hand-suckered check and those of a contact substance applied at the early flower stage followed by a systemic chemical applied from 1 to 2 wk after the contact chemical (sequential). For burley tobacco, the specified chemical and physical properties should fall between those of the standard hand-suckered check and a maleic hydrazide treatment. If the data are statistically analyzed, the 0.05 level of significance will be used to determine if the specified chemical and physical properties fall between the standards.

4. In the warehouse evaluation, the cured leaf from treated plants must compare favorably with the standard hand-suckered and sequential treatments for the flue-cured tobacco and with the standard hand-suckered and the maleic hydrazide treatment for the burley tobacco.

5. Smoke-taste tests are designed to ascertain if the chemical treatment imparts an off-taste to the smoke of cigarettes manufactured from treated tobacco. They are to be conducted as comparison tests between a known check sample and a treated sample. A coded check sample will be used as an internal standard. In addition to the simple evaluation of "acceptable" or "not acceptable," the percentage of acceptable and non-acceptable and the number of panelists will also be reported by each tobacco company making smoke panel tests. Each company is to specify which treatment, if any, is considered objectionable.

Company Proprietary Tests. The chemical company sponsoring a chemical is responsible for conducting these final "on farm"-type tests. All data is to be submitted to the Regional Committee for evaluation. General requirements include:

1. Using the same formulation tested in the advanced and/or preliminary tests, the company is to conduct field tests at a minimum of 3 flue-cured and/or 2 burley tobacco locations.

2. Applications should be made with mechanical sprayers used in the commercial production of tobacco. More than 1 application rate also is desirable.

Field test procedures and data requirements for company proprietary tests include the following:

1. Distribution and test locations. For flue-cured tobacco, 1 southern (Fla., Ga., or S.C.); 1 eastern (N.C.); and 1 old belt (N.C., Va.). For burley tobacco, 1 high elevation (N.C., Va.) and 1 lower elevation (Tenn., Ky.).

2. Plot size. 1/10 acre as single or replicated small plots. Data taken on 3 10-plant subsamples. One 30-plant topped, not suckered, plot is to be included for calculation of percent suckered control.

3. Agronomic data.

a. Efficacy based on percent suckered control, sucker number and sucker weights.

b. Phytotoxicity at maximum manifestation (0 to 5 scale).

4. Residue determinations on cured leaf (to be reported to Committee for informational purposes only). (See regional advanced test.)

5. Modified chemicals and/or formulations going through company proprietary tests which have not been subjected to smoke tests will require such tests.

TEST STANDARDS

1. Sucker control treatments.

a. Contact: Off-shoot-T

b. Systemic: MH-30

c. Sequential: Off-shoot-T followed by MH-30

d. Untreated: hand-suckered (HS) (suckers are removed when they are 4 to 6 in. long); topped, not suckered (TNS).

2. Degree of sucker control measured as total number and percent by which treatment reduced sucker green weight compared with topped but not suckered check (90 percent of appropriate standards).

3. Leaf injury.

a. Scale, 0 to 5: 0=none; 1=very slight; 2=slight; 3=slight to moderate; 4=moderate; 5=severe.

b. No leaf drop compared to appropriate standards.

4. Warehouse evaluations by tobacco companies based on percent usable tobacco (average and total usable by at least 1 company).

a. Regional preliminary test requires percent usable only.

b. Regional advanced test requires percent usable and body and texture for flue-cured tobacco; percent usable and quality rating for burley tobacco.

5. Chemical and physical properties must fall between the properties of standard hand-suckered and sequentially treated flue-cured tobacco, and between the properties of standard hand-suckered and MH–30 treated burley tobacco.

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CONTROL OF POTATO TUBER SPROUTING

In recent years, several types of growth regulator applications have been used to successfully induce dormancy: (1) Applying the product as preharvest applications; (2) dipping tubers in a solution of the product before storage; (3) dusting the tubers with the product before storage; (4) applying the regulating chemical as a volatile suspendable solid to the tubers while in storage; or (5) applying the product as a spray or wax solution to the tubers as they are packaged for the retail market. Factors, such as storage temperature, affecting potato sprout inhibition and methods of testing have been discussed in several publications (Sparks and Summers, 1974; Zaehringer, et al., 1966; Rao and Wittwer, 1955; Hruschka et al., 1965; Nylund and Ayres, 1964; Cunningham et al., 1966; and Weaver, 1972).

Test Conditions. The usual procedure is to randomly collect about 400 tubers for each variable to be tested. They should be field-run quality but with no green, rot or serious malformations. These are divided into 4 replicates of 100 tubers each. The method of applying the inhibitor depends upon the time at which the material is to be applied. Several dosage levels of the chemical under test are necessary.

In the case of preharvest applications of growth regulators to potato plants, variables, such as time of application, dosage of chemical applied, time of harvest and effect of rainfall must be considered in addition to subsequent storage conditions. Replicated plots (usually 4 replicates) sufficiently large to allow statistical analysis should be used.

Treated tubers or tubers from treated plants should be stored at various temperatures, subjected to various airflows and treated with air of different humidity levels to determine length of control (Sparks, 1965 and Sparks, 1973). Retreatment with the inhibitor may be required if the material is volatile and high rates of airflow are used. For comparison purposes, untreated tubers and tubers treated with standard sprout inhibitors are to be included. Standard chemicals may include CIPC (isopropyl-m-chlorocarbanilate), TCNB (1,2,4,5-tetrachloro-3-nitrobenzene), maleic hydrazide or other chemicals registered for this use.

Interpretation of Test Results. At monthly intervals, for 12 mo, data on weight loss, rot and sprouting should be taken. At the end of the storage period other data are also usually obtained:

1. Weight loss.
2. Rot loss.
3. Sprouting.
4. Flattening and shriveling.
5. Effect on suberin and wound priderm formation.
6. Heat generated in the potato pile (a gross measure of respiration rate).
7. Quality change (sugars, mealiness, fry color, flavor, chipping).
8. Appearance change (loss of net or russetting).
9. Utilization as propagation stock.

References

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CONTROL OF BRANCH ANGLE IN YOUNG TREES

In many trees, especially apples and pears, the primary branches form narrow angles with the main trunk which result in structurally weak crotches that often break under moderate or heavy fruiting and seriously reduce the productiveness of the tree. Also, such narrow crotches are very prone to winter injury. Within a few years the entire tree may be lost from further weakening and from secondary infection. The development of wide branch angles avoids the bark inclusion in the tree crotches and the trees can bear heavier crops when they begin fruiting. The more open growth also permits more light into the center of the tree, which is particularly useful on spur type Delicious apples or regular Delicious on dwarfing stock. Factors affecting chemical treatments to modify crotch angle and methods of testing to demonstrate the efficacy of a chemical treatment have been discussed in several publications. (Verner, 1938; Bukovac, 1963, 1968).

Test Conditions. Young fruit trees, usually in the second or third year after being set in the field, are sprayed with several concentrations of the test chemical. The correct timing of application must be determined (usually when the new shoot growth is 3 to 5 in. long). The angle at which the branch leaves the main stem should be measured at the end of the growing season. The effect on vegetative growth also should be measured. Some of the factors affecting product performance are listed below.

1. Volume of spray may be important. The trees should be sprayed to runoff until sufficient data show that lower volumes can be used.

2. Since response to chemical treatment can vary widely with variety the effect on all varieties should be studied.

3. Tests should be made for each variety to determine the rate (lb. active ingredient/100 gal of spray solution and *volume* of spray solution applied per tree) and the stage of development of the tree at the time of application that will give the best response under the environmental conditions where the product will be used.

4. The effect of seedling vigor and rate of growth should be determined, especially in relation to inhibition of vegetative growth.

5. The effect of rainfall following application should be determined.

6. The effect of a repeat application the following year should be determined.

Interpretation of Test Results. To support label claims for modifying crotch angle development in fruit trees, the treatment should increase the angle at which the branch leaves the main stem. This is more important than just causing the outer part of the branch to bend downward. In general, the angle should be greater than 45 degrees to insure wide angle laterals for a sounder structural framework in trees.

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CONTROL OF ALTERNATE BEARING IN APPLES

Many varieties of apples bear heavily 1 year ("on" year) and very little the following year ("off" year). Chemical treatment (return bloom treatment) applied in the "on" year before blossom differentiation can increase yield during the "off" year. In addition to leveling out crop production for the 2-yr cycle, such treatments often result in an increase in total yield for the period.

Test Conditions. A series of test trees, in the "on" year, should be paired on the basis of uniformity of rootstock, tree size and the intensity of blossoming in the spring of the year. One tree of each pair is sprayed with the chemical treatment; the other should remain untreated. Each treatment should be sprayed to runoff until data are accumulated to show that lower volumes are equally effective. Various rates need to be tested on each variety to determine the optimum rate. Timing of application must also be determined. (The optimum timing usually is 4 to 5 wk after full bloom.) To maintain uniform crop production, it is necessary to treat only during the "on" year. Because the degree of blossoming in the spring will determine those years in which treatments should be made, studies are needed on each variety on the degree of spring blossoming and the amount of return bloom the following season.

Since climatic conditions can affect performance, it is absolutely necessary to test the compound in each location where it will be used. Growers should be cautioned to start with a small block of trees until they gain experience in the performance of the compound under their environmental conditions. Any affect of rainfall following application should be determined.

The effect of a blossom thinning treatment or the occurrence of frost on blossom set should be determined before the application of the return bloom treatment. Studies with various degrees of blossom thinning are needed to determine the rate and effect of the return bloom treatment.

Tree vigor and size, as well as the mineral nutrition of the tree, also can affect response.

Interpretation of Test Results. The effect of any treatment on the control of biennial bearing will be noted in the year following treatment. The yield should be taken at the end of the second season and compared with untreated trees. However, since the objective is to even out the productivity of the tree, yields should also be taken at the end of the first season and the total yield for the 2 yr should be compared.

Normally, yield alone will not give the total picture and it is necessary to grade the fruit and determine the market price for each grade. This may show a greater cash return to the grower than if total yield alone were the only criterion used. Return bloom treatments may also inhibit vegetative growth, and an additional benefit may be a reduction in the amount of pruning necessary. There is also a possibility of delayed bud growth in the spring which can be helpful to the grower in areas of late spring frosts.

Some other effects which should be reported may include: (1) The treatment can hasten or retard the maturity of the crop in the treatment year; and (2) the treatment may increase the severity of a physiological disease known as "bitter pit."

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CONTROL OF SEX EXPRESSION IN CUCUMBERS AND SQUASHES

The biochemical control of sex expression in cucumbers and squashes aids in the production of hybrid seed and also can be useful in increasing yield, especially where once-over destruct harvest is practiced. A chemical treatment is made at the early seedling stage of growth to increase the number of pistillate (female) flowers and to decrease the number of staminate (male) flowers. These treatments bring about formation of female flowers at lower nodes where normally only male flowers are formed in the standard (monoecious) cucumber and squash varieties. Factors affecting this sex response and methods of testing to demonstrate the efficacy of a chemical treatment have been discussed in a number of publications (McMurray and Miller, 1968 and 1969; Robinson, et al., 1969; Rudich et al., 1969; Sims and Gledhill, 1969; Weaver, 1972).

Test Conditions. For sex ratio studies, field trials consisting of single-row plots 8 ft long with 10 plants per plot are adequate. Each treatment and control plot should be replicated 4 times. For yield trials, however, single-row plots 40 ft long with 120 plants per plot should be used, with 6 replications per treatment. The test plots should be located in the middle of a field containing sufficient untreated monoecious plants to assure an adequate supply of pollen. Application should be made to seedling plants to assure good coverage (100 gal/acre).

To evaluate the effectiveness of a treatment on sex expression, the sex of each flower at each node should be determined on a weekly basis during the blooming period. To determine the effect on yield, the plots should be harvested twice a week, starting when the first fruit is at the proper stage. For mechanical harvesting, each plot should be harvested separately at the correct stage to take into account any delay or hastening of maturity. In all cases, the yield should be separated by grade and the market price for each grade at each harvest date should be determined.

Some of the test conditions and variables that should be studied are listed below.

1. For hybrid seed production the amount of seed per fruit should be determined. Also, the percentage germination and the storage life should be studied.

2. The response between varieties can differ significantly, and the correct timing, rate and number of applications should be studied on each variety under different environmental conditions.

3. The effect on delay or hastening of maturity should be determined.

4. Effects on fruit quality and fruit shape should be made.

5. Since the effect on sexual modification towards femaleness in the treated plants may be temporary, studies should be made to determine the length of effectiveness of each treatment.

6. The effect of rainfall following application should be determined.

7. The effect of the treatment on vine growth should be noted.

8. Any effect on time of flowering should be noted so that the pollination line can be planted at the proper time to supply adequate pollen.

Interpretation of Test Results. To support claims as an aid to hybrid seed production, the data should show an increase in the percentage of female blossoms at the early stages of flowering. Seed production in the treated fruit should not be decreased, and the hybrid seed should have good germination and storage life.

To support claims for increased yield, data are needed for yield and grade for the period during which the crop is harvested and marketed. Total yield during the growing season is often very misleading, especially if a large percentage of the fruit falls in the low-priced grades. In once-over destruct harvests, comparisons are needed between yield from treated and untreated control plots harvested at the same stage of development. If the chemical treatment hastens or delays the development of the fruit, it is necessary to harvest the treated and untreated control plots at the same stage of development but maybe at different times. The chemical treatment should not cause any adverse effect on fruit shape or quality.

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CONTROL OF FRUIT MATURATION AND RIPENING

Growth regulators are being tested experimentally and used commercially to achieve a number of specific objectives related to fruit maturation and ripening. Included in these objectives are (1) enhancing pigmentation in cherries, cranberries and apples (Chaplain and Kenworthy, 1970; Eck, 1969; Edgerton and Blanpied, 1968); (2) hastening or delaying maturity in peaches, tomatoes and apples (Looney, 1972; Robinson et al., 1968; Unrath, 1971); (3) the concentration of ripening in tomatoes, blueberries and apples (Iwahari and Lyons, 1970; Eck, 1970; Schomer et al., 1971); (4) improving uniformity of ripening and enhancing fruit quality in bananas (Russo et al., 1968); and (5) enhancing abscission and loosening to facilitate mechanical harvesting (Bukovac et al., 1969, 1971).

Test Conditions. Test conditions that should be reported are plot sizes, number of plants per plot, number of replications and controls. Information on product application should include number of treatments; rates of application; time of application; methods of application; a comparison of dilute versus concentrated applications and low versus high gallonage applications; and possible interaction with other chemicals applied. Differential varietal responses are important and should be adequately investigated.

Possible parameters in measuring growth regulator effects on the maturation and ripening process include such quality measurements as sugars, acids, pigments, viscosity, firmness, dry weight, flavor, appearance, short and long term storage quality and size. Yield, also an important parameter, might include measurement of total yield, marketable yield, grades, percent of crop mature at a single harvest and yield distribution over the entire harvest period. Residual effects should also be evaluated, particularly as they may influence subsequent flowering and fruit set on perennial plants and seeds of annuals.

Some other conditions that should be studied are listed below.

1. Plant condition should be monitored, noting particularly any possible stress conditions.

2. Environmental conditions, particularly temperature, soil moisture, and possible weather conditions before, during and after treatment, should be monitored.

3. Soil residual effects on greenhouse and container grown crops should be measured.

4. Changes in insect and disease susceptibility should be noted.

5. Any chemical residue on the harvested fruit should be measured.

Interpretation of Test Results. Suggested interpretations in terms of the quantitative

level of response obtained from growth regulator treatment might include: (1) A 15 percent improvement in yield, when yield is the primary objective; (2) advance or delay of harvest by a week for a growth regulator treatment designed to modify the harvest season of a particular crop; (3) a pigment enhancement of 50 percent, particularly in early harvested fruit; and (4) concentration of yield which results in 80 percent of the total harvest occurring in a single picking for a growth regulator designed to concentrate the harvest.

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CONTROL OF FRUIT ABSCISSION

Soon after mechanical harvesting of fruits was tried, it became obvious that, in many cases, treatment of the trees or vines with abscission agents (chemical looseners) would be beneficial and that, with some types of mechanical harvesting equipment such as air harvesters, it is required (Wilson and Coppock, 1968; 1969). Citrus, cherries, olives, apples, grapes and pecans are examples of crops whose harvest can be aided by the use of such compounds (Weaver, 1972). Specific requirements for each crop may vary somewhat, but the overall principles involved appear to be similar. Although the following test conditions relate primarily to citrus fruits, some examples of problems encountered with other crops are also included.

TEST CONDITIONS

Method of Application. The application method usually will be by dilute or concentrated spray. Most of the current abscission chemicals under investigation appear to function through contact rather than systemic action; a uniform spray coverage of trees and vines, therefore, is necessary. Other methods of application should be evaluated as to their practicality under field conditions (Wilson, 1966; 1967).

Timing and Environmental Conditions. Most abscission agents produce fruit loosening within 3 to 7 days following spray application although this may vary depending on the crop involved. With citrus, the abscission agent can normally be applied at any time during the period when the fruit is considered legally mature (Wilson, 1969)

Weather conditions may affect abscission chemicals (Wilson, 1969; 1972) and should be studied. Although no loss of activity due to high temperatures has been reported, abnormally wet and cold conditions may adversely affect results either by washing off the material before it has time to act or delaying or reducing its effects by slowing metabolic processes.

Consistency of Performances. Failures caused by adverse environmental conditions can occasionally be expected; unexplained failure occurring under good environmental conditions, however, should be rare. Thus, inconsistency in chemical performances should usually be interpreted unfavorably. The effects of various rootstocks should also be considered in evaluating results, for, with citrus fruits, certain rootstocks produce fruit which loosen more readily when abscission chemicals are applied (Cooper and Wilson, 1971).

Physical Measurements of Chemical Effectiveness:—The most common method used to determine degree of citrus fruit loosening is through use of a pull force measuring device (Hendershott, 1964). Most of these devices measure straight pull breaking force necessary to separate the stems from the fruit. These pull forces may vary from a few grams (olives) to 30 to 40 lb (some citrus fruits). A measure of effectiveness is a substantial reduction in the average pull force measurements from that of a control.

Interpretation of Results. In evaluating abscission results, 2 principal factors must be considered, depending on which of the methods of removal discussed below is employed and the ultimate use of the fruit (processing or fresh).

If fruit is to be shaken to the ground for mechanical or hand pickup, maximum fruit loosening is desired. Under these conditions, there is a high probability that substantial fruit drops will occur before harvest (Wilson, 1973). With citrus fruits, pull force will be reduced from 14 to 18 lb for untreated fruit to an average of 2.5 lb for treated fruit. Fruit drops of 20 to 50 percent could be expected when fruit is this loose. Under some experimental conditions and sometimes under field conditions, fruit loosening will be so complete that the fruit will separate from the stem when touched and prevent the measurement of pull force. These results can be classed as "too loose to pull," "all fruit dropped" or some other appropriately descriptive phrase.

If fruit is to be shaken by a mechanical device onto a catchframe, fruit loosening should not be as complete as above. A chemical developed for use with catchframes should loosen citrus fruit ideally to no less than 6 lb average pull force.

Abscission agents are of 2 types: (1) Chemicals which release ethylene or cause ethylene production by the fruit without peel injury; and (2) chemicals which may cause superficial rind injury with resulting wound ethylene formation. Although abscission not related to the action of ethylene may be possible, all present commercial abscission agents appear to utilize the ethylene mechanism. Many fruits, including citrus fruit intended for fresh fruit utilization, apples, cherries, and perhaps olives and pecans (nuts) respond to chemicals with this mode of action.

Citrus fruits loosen readily when chemicals cause superficial peel injury. Chemical damage to the rind is acceptable on citrus fruit for processing if it does not cause fruit splitting and excess rotting under wet conditions (Wilson, 1972) or adversely affect juice flavor.

The amount of phytotoxicity which can be tolerated by trees and vines before yields and/or fruit quality are adversely affected varies with the crop and time of year. No specific percentages of leaf drop have been determined for citrus fruits which indicate the upper limits of phytotoxicity; experience has shown that a fairly large margin of safety is necessary. With a crop, such as apples, excessive leaf drop accompanying abscission sprays applied in the fall occurs then anyway. With some other crops, economic considerations may be such that some phytotoxicity can be tolerated.

Because mature fruits, leaves, immature fruits and flowers are all subject to abscission through basically the same physiological process, the growth regulator(s) selected for abscission should be specific to removal of only mature fruits or nuts. The Valencia orange, for example, poses a distinct harvesting problem because its lengthy development period (11 to 18 mo) results in the presence of flowers or immature (green) fruits during most of the harvest season. It is important, therefore, that any abscission chemical selectively loosen mature fruit but cause minimum phytotoxicity to flowers, immature fruits or young growth which might be present.

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INCREASED BERRY SIZE OF SEEDLESS VARIETIES OF TABLE GRAPES

When vines of Thompson or other seedless grapes are girdled at the fruit-set stage, a large increase in berry size—sometimes as much as 100 percent—usually results. Since about 1960, almost all Thompson Seedless grapes intended for table use have been sprayed with 1 or 2 applications of gibberellin. These treatments, along with girdling, increase berry size in a uniform manner (Weaver, 1972).

Test Conditions. The test chemicals are usually applied after shatter (separation of calyptra from flower) following bloom (also the normal girdling time). Four vines per replicate should be sprayed to wet cluster area thoroughly. Use 5 concentrations of the test product and compare results with untreated controls and with gibberellin at label rates.

Test Results. At harvest the following measurements should be made:

1. Crop weight per vine.
2. Average weight of 100 berries per vine to indicate berry size. (Take berries from various clusters and from base, middle and apex of cluster.)
3. Brix value of juice taken with a refractometer.

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LOOSEN COMPACT CLUSTERED VARIETIES OF VITIS VINIFERA (WINE GRAPES)

The incidence of rot in seeded wine grapes is excessive in varieties that produce compact clusters, for example, Zinfandel and Carignane. Berries are pushed off by other expanding berries, and the juice from the fallen fruit fosters decay organisms. Tight clusters are also slow to dry after rains. Loosening of the cluster is a result of reduced set, cluster elongation, and/or production of shot berries (Weaver, 1972); prebloom sprays of gibberellin also loosen these clusters (Weaver and McCune, 1959).

Test Conditions. Test chemicals are usually sprayed onto the vines 2 to 3 wk before bloom when the shoots are 15 to 20 in. long; clusters should be 3 to 4 in. in length, but may range from 2 to 5 in. in length. Use 4 replicates of 4 vines per treatment. Use 5 concentrations of the test product and compare results with untreated controls and gibberellin at label rates for the variety. The clusters must be thoroughly wet.

Test Results. At harvest the following measurements should be made:

1. Estimate of looseness percentage. The compact control clusters are considered to have 0 percent unoccupied space. Estimate that part of the total volume of a cluster not occupied by berries.
2. Number of berries per cluster (for the determination of effect on berry set).
3. Length of cluster from basal lateral to the apex of the cluster.
4. Berry size (average weight of 100 berries).
5. Degrees Brix value of juice taken with a refractometer.
6. Percentage of rot which has occurred in each treatment.

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ACIDITY REDUCTION IN CITRUS

The 2 main purposes for acidity reduction in citrus fruits are to produce early maturity of fruit and thereby lengthen the marketing season and to produce sweeter fruit. In analytical chemical terms, "sweet" means "high ratio fruit" as measured by the standard accepted Brix/acid determination. Maturity of citrus fruits generally follows the pattern of gradual reduction of acid during the fruit season, and usually there is a corresponding increase in soluble solids (sugars).

Test Conditions. Field trials to reduce acidity may consist of single-tree plots replicated 6 times. Materials are applied as sprays to thoroughly cover fruit and foliage. With citrus fruits which maintain a generally recognized low respiration rate, the time of application of the sprays is usually 4 to 10 mo before harvest. (The development of sprays which would shorten this interval would be desirable.) At the present time maturity sprays are applied from postbloom until the end of the summer spray period, although the most effective time is 1 to 6 wk following bloom—late March to early May.

Twenty fruit per tree, a total of 120 fruit per treatment picked at random (inside and outside of the canopy), are taken from the treated, control and standard product at biweekly intervals 30 days before and during the normal harvest period. The following measurements should be taken:

1. Determine the percent juice for each sample (total weight of squeezed juice per unit weight of fruit).
2. Determine the percent acid (total titratable acid) using phenolphthalein as the indicator and 0.3125 *N* NaOH as the basic solution.
3. Determine Brix value for each sample.
4. Calculate ratio of Brix value to acid.

Interpretation of Test Results. At least 2 limitations to use of an acidity reducing compound should be considered:

1. Oranges, tangerines, grapefruit, tangelos and tangelo hybrids can be subject to excessive acidity reduction and produce a sweet fruit with an undesirable "inspid" flavor.
2. "Flavor" of a fruit or fruit juice is made up of several components. Although a growth regulator may reduce juice acidity of immature fruit to what would be considered acceptable for mature fruit, it would not affect the raw, immature flavors also present in the immature fruit. Normally these immature flavors diminish at a Brix of 9.0 or higher.

APPLE SCALD INHIBITORS

Substantial information about scald is available, as it has been widely studied for over 60 yr (Huelin, 1964; Smock, 1961; Smock and Southwick, 1945). The cause is still unknown or indefinite. Because it is usually much worse on early pickings, it is associated with immaturity. Scald susceptibility varies from season to season so weather before harvest also is a factor. Many researchers believed that volatile materials caused scald, since relatively high levels of esters induce an artificial scald-like injury on apples, but the volatile theory is no longer widely accepted. Recently, Australian researchers (Pierson et al., 1971) have suggested that oxidation products of α-farnesene are the direct cause of superficial scald. They further suggest that effects of artificial antioxidants on the oxidation of α-farnesene could first be measured in comparatively short tests before testing for control of scale on apples.

For many years use of tissue wraps containing mineral oil around each fruit was the standard scald-control procedure. Growth regulating substances, such as naphthalene acetic acid (NAA), applied as a postharvest dip provided considerable control but were not used commercially (Schomer and Marth, 1945). Commercial scald control is now obtained by using 1 of 2 anti-oxidants (Smock, 1961); diphenylamine or 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin). Both chemicals are registered for use on apples and provide good scald control (Dewey and Dilley, 1964; Hardenburg and Anderson, 1962; Huelin, 1964; Mattus and Rollins, 1963).

TEST CONDITIONS

1. Use freshly harvested apples, 50 to 100 per replicate. More scald will develop in control lots if fruit is picked 1 wk before the best commercial date. McIntosh, Rome Beauty and Red Delicious are scald-susceptible varieties.
2. Apply test product and appropriate standard products within the first 7 to 10 days after harvest. Dip or submerge apples for 10 sec in test solutions or suspensions. Keep test suspensions well-agitated.
3. Fruit temperature and treatment solution temperature should be moderate (60° to 70° F). Cold fruit will not take on a good coating of test chemicals.
4. After treating fruit, tilt boxes or turn fruit to improve drainage and prevent possible chemical burn in stem or calyx cavities. Examine for initial visible residue.
5. To predict scald intensity, store treated and untreated fruit samples in boxes with unsealed 1.5-mil polyethylene liners for 4 to 6 wk at 70°F (Smock, 1961).
6. To determine commercial scald control, store treated and untreated fruit samples in unlined boxes for 5 to 6 mo at 32°F (38°F for McIntosh) with high relative humidity (90–95 percent), then transfer to 70°F for 7 days to allow scald symptom development (Hardenburg and Anderson, 1962; Huelin, 1964; Mattus and Rollins, 1963; Smock, 1961).

INTERPRETATION OF RESULTS

1. Determine the number of fruit with no scald, slight scald and severe scald. Slight scald is that affecting 10 percent or less of the skin surface and is light brown in intensity. Severe scald is that affecting more than 10 percent of the surface or is dark brown and markedly disfiguring.
2. Determine flesh firmness of fruit from treated and control fruit samples using a fruit pressure tester to determine any adverse effect on ripening or softening rate.
3. Examine fruit for "off odor," "off flavor," visivle residue, lenticel injury or chemical burn symptoms.

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SUBPART E

Product performance: Invertebrate control agents IV.E–1
Foliar treatment IV.E–1
Fruiting vegetables IV.E–1
Ornamental foliage plants (private and commercial dwellings) IV.E–1
Greenhouse ornamentals IV.E–1
Livestock, poultry, fur and wool-bearing animals IV.E–1
Dairy animals as produce IV.E–1
Fur and wool-bearing animals as produce IV.E–2
Meat animals for slaughter IV.E–2
Miscellaneous animals (not intended for slaughter) IV.E–2
Premise treatments IV.E–2
Household IV.E–2
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PART IV.—PRODUCT PERFORMANCE

SUBPART E.—INVERTEBRATE CONTROL AGENTS

The methods presently referenced in this subpart include only bioassay methods developed by the Chemical Specialties Manufacturers Association (CSMA). They are acceptable for deriving data for product registration in laboratory or greenhouse testing. No field-testing methods or protocols are included at this time.

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Fruiting Vegetables. CSMA. 1971. Determination of plant safety of pressurized formulations for use on plants in the house or garden. *Soap Chem. Spec. Blue Book.* 47(4A): 173–174.

Ornamental Foliage Plants (private and commercial dwellings). CSMA. 1971. Determination of plant safety of pressurized formulations for use on plants in the house or garden. *Soap Chem. Spec. Blue Book* 47(4A): 173–174.

Greenhouse Ornamentals. CSMA. 1971. Determination of plant safety of pressurized formulations for use on plants in the house or garden. *Soap Chem. Spec. Blue Book.* 47(4A):173–174.

LIVESTOCK, POULTRY, FUR AND WOOL-BEARING ANIMALS

Dairy Animals as Produce. CSMA. 1971. Evaluation of field performance of oil-based livestock sprays. *Soap Chem. Spec. Blue Book.* 47(4A):172, 190.

Fur and Wool-bearing Animals as Produce. CSMA. 1971. Evaluation of field performance of oil-based livestock sprays. *Soap Chem. Spec. Blue Book.* 47(4A):172, 190.

Meat Animals for Slaughter. CSMA. 1971. Evaluation of field performance of oil-based livestock sprays. *Soap Chem. Spec. Blue Book.* 47(4A):172, 190.

Miscellaneous Animals (not intended for slaughter). CSMA. 1971. Evaluation of field performance of oil-based livestock sprays. *Soap Chem. Spec. Blue Book.* 47(4A):172, 190.

PREMISE TREATMENTS

Household. CSMA. 1971. Determination of plant safety of pressurized formulations for use on plants in the house or garden. *Soap Chem. Spec. Blue Book.* 47(4A):173–174. CSMA. 1971. Cockroach spray method. *Soap Chem. Spec. Blue Book.* 47(4A):166–167. CSMA. 1971. Cockroach aerosol test method. *Soap Chem. Spec. Blue Book.* 47(4A):164–165, 191. CSMA. 1971. The Peet-Grady method: official method of the CSMA for evaluating liquid household insecticides. *Soap Chem. Spec. Blue Book.* 47(4A):158–160. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191. CSMA. 1971. Textile resistance test. *Soap Chem. Spec. Blue Book.* 47(4A):168–171.

Commercial Storage and Transportation Facilities. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Hospitals. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Commercial, Industrial and Institutional Maintenance. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Food Processing Plants. CSMA. 1971. Cockroach spray method. *Soap Chem. Spec. Blue Book.* 47(4A):166–167. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Eating Establishments. CSMA. 1971. Cockroach spray method. *Soap Chem. Spec. Blue Book.* 47(4A):166–167. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Animal Feedlots and Holding Pens. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Farm Poultry Houses and Yards. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A):161–163, 191.

Farm Dairy Structures. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A): 161–163, 191.

Animal Buildings (other than dairy or poultry). CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A): 161–163, 191.

Farm Storage Structures and Equipment. CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A): 161–163, 191.

Greenhouses and Mushroom Houses (empty). CSMA. 1971. Aerosol and pressurized space spray insecticide test method for flying insects. *Soap Chem. Spec. Blue Book.* 47(4A): 161–163, 191.

STORED PRODUCTS

Stored Raw Agricultural Food and Feed (except grain). CSMA. 1971. Cockroach spray method. *Soap Chem. Spec. Blue Book.* 47(4A): 166–167.

Stored Food and Feed Products. CSMA. 1971. Cockroach spray method. *Soap Chem. Spec. Blue Book.* 47(4A): 166–167.

FABRICS

Laundry and Dry Cleaning. CSMA. 1971. Textile resistance test. *Soap Chem. Spec. Blue Book.* 47(4A): 168–171.

Commercial and Industrial Products. CSMA. 1971. Textile resistance test. *Soap Chem. Spec. Blue Book.* 47(4A): 168–171.

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PART IV.—PRODUCT PERFORMANCE

SUBPART F.—VERTEBRATE CONTROL AGENTS

This subpart represents an appraisal of current methodology for evaluating the efficacy of vertebrate control agents. The classes of agents scrutinized are toxicants, repellents (including aversive conditioners), reproductive inhibitors, anesthetizing chemicals and devices. The *mode of action* of the control agents could not be examined on an agent-by-agent basis, especially in respect to the chemical senses. The vertebrate species include amphibians, reptiles, birds and mammals. The text is arranged in phylogenetic order, with any necessary introductory explanation about the methods preceding each target species. (For fish, see Part IV.B., Aquatic Pest Control Agents, and Part VII.B., Aquatic Toxicology.)

The appraisal primarily evaluates, or references, methods that have been tested and published in journals and technical bulletins; some information was derived from unpublished reports and manuscripts. The fact that only limited research has been conducted to test many vertebrate control agents accounts for the paucity of citations in some areas. Also, control agents for some species, such as bears, have not been extensively studied because the damage caused by the animal to human property or interests is limited.

Most target groups lack well documented, sound experimental protocols at this time. A great deal more research is needed in some areas before complete, acceptable testing protocols can be defined.

AMPHIBIAN TOXICANTS AND REPELLENTS

Many repellents and toxicants for amphibians could be tested following the methods used for fish, since in most cases problems caused by them are associated with fish production. Tadpoles, for example, are no problem where there are bass present in ponds, but in hatchery situations they may multiply rapidly and interfere with the growth of desirable fish (Helms, 1967). There are some notable exceptions; i.e., native toads have been reported to eat bees in apiaries in large numbers in California (Eckert, 1934).

Laboratory Studies. It is necessary to know the complete life history of the particular amphibian which one wishes to control, since the laboratory studies would vary according to the phase of the life cycle the toxicant or repellent is to control. Testing to determine LC–50 values at 24, 48 and 72 hr (Helms, 1967) was done in 15-gal. aquaria. Three to 5 test animals were placed in each aquarium and then the test chemical was added. A sixth aquarium was maintained as a control. Bullfrog and leopard frog tadpoles and a few species of toad tadpoles were tested. Seven species of fish were also tested under identical conditions.

Field Studies. After testing in aquaria, Helms conducted field studies. The methodology varied depending on the life history of the organism. In the case of tadpoles, 9 treatments were carried out in 6 different ponds. Following treatment, the tadpole numbers were checked by seining to determine the completeness of kill and whether nontarget species were affected.

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REPTILE TOXICANTS AND REPELLANTS

The reptiles include the skinks, horned toads, alligators, turtles and snakes. The lizards seldom need control, and turtles can be controlled by trapping, fishing or shooting (Dillard, 1973). Alligators are protected by Federal law.

Many snakes are beneficial or neutral because they consume rodents and insects. With about 400 deaths per year from snakebite in North America, and worldwide, about 50,000 deaths per year (Swaroop and Grab, 1954), however, some reptiles may require control.

Toxicants. No test methods for reptile toxicants were identified for review.

Repellents. Some of the literature points out that repellents may be acceptable control measures. Cowles and Phelan (1958) have shown that snakes are very sensitive to odors. Crawford and Gross (1969) have shown that snakes show heart and respiration rate responses to chemical stimuli.

Porter and Czaplicki (1974) have shown that both water snakes and garter snakes are sensitive to chemical cues produced by themselves or other species. The authors conducted a series of experiments to test the attractiveness or the repellency of snakes to others of the same species or other species.

The first set of experiments was carried out with small snakes (less than 17.7 in.). Ten glass aquaria with slate bottoms and porous lids were used. These were divided in half by tightly fitting partitions. The stimulus snake was confined to one side for 47.5 hr. All snakes defecated in their halves during the exposure period. At the end of the confinement the snake and partition was removed. A strip of tape was used to mark the bottom where the partition had rested. One test snake was then laid on the tape and the side (soiled versus clean) to which it moved was recorded. The snake's position was noted every 30 min throughout an 8-hr testing period. When using wild-caught garter snakes, the authors employed larger testing chambers. These were divided in the same manner and procedures were the same.

Jenkins' (1962) method for screening was to place 3 pairs of boxes, each box with one open end, in pens. The tops of the boxes were removable and the floors were covered with soil. The boxes, similar to squirrel live traps, were placed at right angles to the axis of the pen. Two openings faced in the same direction for each pair. This direction was opposite to that of the adjacent pair. Every other box was used as a control. All snakes were marked, for ease of identification, and placed in the ends of the pen farthest from the boxes and observed.

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AVIAN TOXICANTS, REPELLENTS, REPRODUCTIVE INHIBITORS AND ANESTHETIZING CHEMICALS

No specific laboratory or field test protocols exist for the efficacy evaluation of avian toxicants, repellents, reproductive inhibitors and anesthetizing chemicals. Recent publications by Lee (1970), Ochs (1972) and Schafer and Guarino (1970) provide brief descriptions of, and the rationale behind, some tests. Since many of the laboratory tests necessary to establish efficacy are described in detail in Part VII of this Appendix (Hazard Evaluation, human and domestic animal toxicity data and fish and wildlife toxicity data), this discussion will elaborate on laboratory tests only when it is necessary to provide additional information relative to avian control. The primary emphasis will be directed toward efficacy determinations in the field.

Discussions relative to laboratory studies for toxicants, repellents, reproductive inhibitors and anesthetizing chemicals have been combined under one heading (Laboratory Methods) since a majority of the laboratory tests must be conducted on all chemicals, irrespective of the proposed use. References to special studies or special emphasis relating to individual proposed uses are made in the text.

Discussions relative to field studies are broken down by the 4 major groups, since evaluation procedures in the field vary considerably. Field test protocols for bird con-

trol agents are still in the developmental stages. Since many of the referenced tests were conducted before the most recent legislative action on FIFRA, as amended, most of them are lacking in one or more of the studies now required. A number of areas can and should be strengthened with regard to all tests:

1. Better knowledge of cultural practices and bird populations in test areas.
2. Use of larger experimental units.
3. Strict adherence to treatment schedules.
4. Use of controls during the same test period.
5. Use of more replications per test.
6. Proper application of statistical techniques.
7. More adequate appraisal of hazards to nontarget species.

It must also be recognized that the field evaluation of efficacy of any bird control agent depends on conducting the test in the proper area at the proper time. Birds and biologists are not always able to mesh their activities in well controlled or ideal test situations; therefore, any proposed protocol must be adaptable to existing test situations.

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AVIAN TOXICANTS, REPELLENTS, REPRODUCTIVE INHIBITORS AND ANESTHETIZING CHEMICALS: LABORATORY METHODS

Acute Toxicity. The various methods for determining acute oral and dermal toxicity in birds are described in Part VII of this Appendix; however, since control agents are seldom developed for use on the test species recommended in Part VII (i.e., ducks and quail), it will be necessary to conduct LD–50 evaluations on the target species *and* on selected nontarget species which may be affected by the proposed control agent. Since availability of wild birds for tests (particularly protected and/or nontarget species) may be severely restricted, it may be necessary to conduct LD–50 determinations with what would normally be considered less-than-sufficient numbers. The moving point interpolation method described by Thompson (1947), Thompson and Weil (1952), and Weil (1952) is particularly appropriate for this purpose. Descriptions of the use of this method for wild birds are provided by DeCino et al. (1966), Schafer (1972), schafer et al. (1973), and Tucker and Crabtree (1970).

Subacute Toxicity. The exact technique used to determine subacute toxicity will depend on the proposed application of the control agent. Tests should be conducted on target and selectd nontarget species. For proposed applications resulting in short-term exposure, the 5-day dietary LC–50 test protocol developed by Heath and Stickel (1965) as modified by Hill (1972) may suffice. Since many applications of control agents (i.e., repellents) may result in longer exposure, longer term tests should be conducted to approximate the maximum period of exposure. The 7- to 40-day test procedures described by Schafer and Marking (1974), Schafer et al. (1974b), and Tucker and Crabtree (1970) may suffice for most applications. It may, however, be necessary to conduct studies for as long as 90 days; in this case the methodology described by Hayes (1967) may be acceptable. Again, the size of test populations will necessarily vary with the availability of the test species.

Inhalation Toxicity. Data on inhalation toxicity will be necessary on the target species and on representative nontarget species if the proposed use of the control agent results in respirable gas, fumes, aerosols or dusts. Because there are no published records of laboratory tests using wild avian species, modified mammalian tests can be used.

Secondary Hazards. Since the use of avian control agents in urban and suburban areas presents a potential hazard to carnivorous avian and mammalian predators, indicative data related to the proposed use should be gathered to define the degree of hazard to canines or felines, at least 1 species of hawk and 1 species of scavenger bird. Schafer et al. (1974a) describes some test methods. Because of the nature of the tests and availability of suitable test animals, small test populations will be acceptable unless hazards are encountered. If hazards are found, more extensive testing may be required.

Repellents. The repellency (or acceptance) of a compound can be determined by a number of different techniques. The initial evaluation may utilize the individual response—no choice method described by Star et al. (1964) and modified by Schafer and Brunton (1971). Although this method usually overestimates the amount of chemical needed to produce a repellent response under field conditions, it does provide data on the maximum treatment levels needed to produce protection. To determine comparative repellency (or acceptance) of a chemical in individual birds and groups on preference, the methodology described by Hill (1972), Luckwill and Weaver (1965), McLean (1972) or Ridsdale and Granett (1969) may be acceptable. Although indications of the repellency (or acceptance) of chemicals can also be obtained when the chemical is incorporated into drinking water (Duncan 1963), tests on foods are preferred unless water is the item to be protected.

Reproductive Inhibitors. Few laboratory methods have been published on wild avian reproduction. Since most wild birds are difficult to breed in captivity, domesticated species have generally been used. The application of a potential reproductive inhibitor to food is the principal treatment method, assuming the proposed application uses oral ingestion. Methodology for determining efficacy in domesticated species are described by Elder (1964), Powell (1966), and Wentworth (1970) for chronic feeding; Jones et al. (1972) and Schafer et al. (1974c) for acute treatment of males; and Wentworth et al. (1968) for the treatment of eggs.

Anesthetizing Chemicals. Laboratory methods for testing a chemical's effectiveness as an anesthetizing chemical are described by Cline and Greenwood (1972), Peek (1966), Schafer et al. (1967), and Schafer and Cunningham (1972).

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AVIAN TOXICANTS—FIELD METHODS

There are 5 published methods that can be used to appraise the efficacy of oral bird toxicants under different application situations: Besser et al. (1967); Crunden et al. (1965); Guarino and Schafer (1967); Royall et al. (1967); and Larsen and Dietrich (1970). Although the methodology described covers basic needs to determine efficacy, more effort needs to be expended with regard to nontarget species.

Acceptable test methodology for determining the efficacy of dermal toxicants, applied as sprays, are readily available: Jackson and Park (1973); Lefebvre and Seubert (1970); Marsh (1964); and U.S. Fish and Wildlife Service (1973). Although these methods may be used for spray applications, they are deficient in information regarding hazards to nontarget species. A single test for contact toxicants is available (Schafer et al. 1969); again, additional data on hazards to nontarget species may need to be provided.

A single published test is available for determining the efficacy of inhalation toxicants (Devore et al. 1966).

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AVIAN REPELLENTS—FIELD METHODS

The determination of the field efficacy of avian repellents is based on 3 main factors—changes in behavior, reduction of bird numbers and a reduction in damage. There are 2 major types of chemical repellents—gustatory and tactile; each requires somewhat different evaluation procedures.

The gustatory repellents, those acting through some physiological mechanism during or after oral ingestion, have been the most thoroughly investigated and appraisals exist for sprouting agricultural seeds (Besser, 1973, Frank et al., 1970, Guarino and Forbes, 1970, Herman and Kolbe, 1971, Ingram et al., 1973, Stickley and Guarino, 1972, West and Dunks, 1969 and West et al., 1969); sprouting horticultural seeds (Abbott, 1958, Royall and Perguson, 1962); and ripening grain (DeHaven et al., 1973, Dolbeer et al., 1973, Guarino et al., 1973, 1974 and Stickley and Ingram 1973). It is particularly important that minimum plot sizes be established according to the recommendations of Guarino (1972) and Herman and Kolbe (1971) and that replications be used.

Other gustatory repellents are those that induce a behavioral response in a very small proportion of birds causing damage. Four published appraisals contain information: Besser et al. (1973); De Grazio et al. (1971, 1972); and Stickley et al. (1972). Again, test plot size and replications are of prime importance.

There are no published acceptable procedures for evaluating tactile avarian repellents.

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AVIAN REPRODUCTIVE INHIBITORS—FIELD METHODS

Only a few tests on efficacy of reproductive inhibitors have been conducted to date. The variety of species evaluated is limited and the adequacy of test methodology in individual studies is not established. Efficacy determinations require measuring fertility rates of treated and untreated birds and effects on breeding behavior, production and population levels.

Test procedures have been developed for evaluating the effects of reproductive inhibitors in various blackbird species (Bray et al., 1974; Fringer and Granett, 1970; Messersmith, 1971; Peek, 1972; and Vandenbergh and Davis, 1962). In addition to the production studies common to each paper, histological evaluations of treated birds (male and/or female) are outlined by Vandenbergh and Davis (1962) and behavioral aspects are described by Bray et al. (1974) and Peek (1972). Effects on nontarget species should also be included in the test design.

Test procedures are less well developed for field evaluations in pigeons (Shortemeyer and Beckwith, 1970; Wofford and Elder, 1967; and Woulfe, 1968) and should be augmented with data on productivity, histology, breeding behavior and effects on nontarget species.

Procedures are also available for gulls (Wetherbee, 1967).

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AVIAN ANESTHETIZING CHEMICALS—FIELD METHODS

The most important measures of the field efficacy evaluation are percent capture, mortality, the time until anesthesia and the duration of anesthesia. For control programs, population reduction by capture and removal is also important.

Methods for evaluating the effects of anesthetizing chemicals on upland game birds have been described by Mosby and Cantner (1956), Williams (1966, 1967), Williams and Phillips (1972) and Williams et al. (1966). However, additional information, such as hazards to nontarget species and body residues in treated birds, may also be required. Accurate scientific description of measurement parameters must also be used (i.e., g/kg bait *not* g/cup).

Methods for using anesthetizing chemicals to capture waterfowl are also available (Crider and McDaniel, 1966, 1967, 1968 and Crider et al., 1968); however, these methods are not complete and additional information may be necessary. Additional methodology is described for other species of birds commonly found near water (Smith, 1967 and Williams and Phillips, 1973).

Methodology for urban pigeon and sparrow control with anesthetics are well detailed by Murton et al. (1963), Ridpath et al. (1961) and Thearle et al. (1971), although certain aspects (e.g., hazards to domestic animals) may need more documentation. Methodology for the use of anesthetizing chemicals in rural situations are best described by Martin (1967). Murton et al. (1963), Ridpath et al. (1961) and Williams and Phillips (1972); however, hazards to predatory animals may need more documentation.

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RODENTICIDES—ACUTE AND CHRONIC

One of the earliest—and relatively comprehensive—publications available on rodenticides and their efficacy is "Biological Methods for the Evaluation of Rodenticides," Bentley, 1958. In 1963, Kverno and Hood published a volume on the evaluation procedures and standards for screening and developing chemicals for forest rodent control.

Laboratory Methods. The Environmental Protection Agency has developed methods that have been used for establishing laboratory efficacy of rodenticides for commensal rodents. (See Exhibits 1–8.)

Articles concerning laboratory test methodology have been published by various researchers: World Health Organization, 1967; Bentley and Larthe, 1959; Dieke and Richter, 1946; Durbin and Robens, 1964; Emlen and Strecker, 1951; Hayes, 1959; Hankins, et al., 1973; Howard et al., 1968; Loosjes, 1959; Saunders, 1955; Savarie et al., 1973; and Ward et al., 1940.

Field Methods. Emlen and Crow (1951), Miller (1953), Richens (1967), Schein (1950), and Wood (1965) have reported on field studies which utilized techniques or methodology of value to those unfamiliar with setting up field evaluations.

The food preferences of the target species, as well as the acceptance or palatability of the prepared bait will have a direct bearing on efficacy in both the laboratory and field. Thus techniques or methods for measuring food preferences or acceptance should be recognized as an integral part of evaluating a rodenticide for any species. Bentley (1958) discussed palatability of baits in considerable detail. His evaluations included measuring the number of visits to the bait, duration of feeding, amount consumed, and rate of intake. He also included comments on the time of onset of poison symptoms and bait shyness.

Shumake et al. (1971) made comparisons of taste preference studies between laboratory and wild Norway rats. Barnett and Spencer (1953) conducted an extensive study of food preferences of wild Norway rats, and Khan (1974) conducted laboratory experiments on food preferences of the black rat. Other researchers, such as Gregson (1966), Cornwell and Bull (1967), Beer (1964), Patric (1970), Fitch (1954), Joy et al. (1967), and Nass and Hood (1969), have published data which may be valuable to those trying to formulate better rodent baits.

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EXHIBIT 1

TENTATIVE ANTICOAGULANT RAT LIQUID BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill rats must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of liquid anticoagulant rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All rats used in this test shall be Norway rats (*Rattus norvegicus*), wild caught or from a wild rat colony; or albino rats (Wistar Strain preferred). Ectoparasite control with 5% Sevin (1-napthyl-N-methylcarbamate) dust is permissible if applied to both test and control animals not less than 7 days prior to start of test. The test rodents should fall within the following sexually mature weight classes (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weight between sexes
Laboratory rat.....	150	300	50
Norway rat.....	175	400	80

3. Pre-test Holding Conditions.

3.1 All rats used in this test method must be held for observation in the laboratory for a period of one week prior to testing. A commercial rat diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50±10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet mix shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners), 5% by weight; Corn oil, 5% by weight. Combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

5. Procedure.

5.1 A test group consists of ten male and ten female rats, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group need be included.

5.2 Provide each cage with a feeder filled daily with the standard TSD challenge diet (4.2).

5.3 Provide each treated cage with equal number of graduated no-drip waterers. Fill half the waterers with tap water and the other half with the test liquid bait formulation diluted with tap water according to use directions. A minimum of 20 ml of each liquid, water and liquid bait, per animal per day should be available. Waterers should be supplied in sufficient quantity to exceed this demand. Replenish liquids as necessary so that both are always equally available to test animals. Reverse position of the waterers daily. The control group shall be provided with graduated no-drip waterers filled only with tap water. Each day record the total quantity of each liquid consumed during the preceding 24 h.

6. Test Period.

6.1 Maintain test period for 15 days, unless a 100% mortality of test rats is recorded prior to that time. Remove dead rats daily or as observed.

6.2 Remove toxicant waterer at the end of the 15-day test period and leave tap-water waterer.

7. Test Period Follow-up.

7.1 Maintain observation on surviving rats for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each rat killed each day during the test period and the five-day post-test period and the amount of liquids consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

EXHIBIT 2

TENTATIVE ANTICOAGULANT MOUSE BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill mice must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of liquid anticoagulant rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All mice used in this test shall be house mice (*Mus musculus*), wild caught or from a wild mouse colony; or albino mice (Swiss-Webster preferred). Ectoparasite control with 5% Sevin (1-napthyl-N-methylcarbamate) dust is permissible if applied to both test animals and control animals not less than 7 days prior to testing. The test rodents should fall within the following sexually mature weight class (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes (grams)
Albino mice	15	[illegible]	5
Wild trapped and colony house mice	10	20	[illegible]

3. Pre-test Holding Conditions.

3.1 All mice used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial mouse diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50 ± 10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed) 25% by weight; Powdered sugar (10 x powdered confectioners) 5% by weight; Corn oil 5% by weight. Combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

5. Procedure.

5.1 A test group consists of ten male and ten female mice, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group need be included.

5.2 Provide each cage with a feeder filled daily with the standard TSD challenge diet (4.2).

5.3 Provide each treated cage with two graduated no-drip waterers, one filled with tap water and the other with the test liquid bait formulation diluted with tap water according to use directions. Replenish liquids as necessary so that both are always equally available to test animals. Reverse position of waterers daily. The control group shall be provided with graduated no-drip waterers filled only with tap water. Each day record the quantity of each liquid consumed during the preceding 24 h.

5.4 Provide shelter in each cage. Use empty soup or beverage cans with one end removed and slightly flattened to reduce rolling. Use one can per five mice.

6. Test Period.

6.1 Maintain test period for 15 days, unless a 100% mortality of test mice is recorded prior to that time. Remove dead mice daily or as observed.

6.2 Remove toxicant waterer at the end of the 15-day test period and leave tap-water waterer.

7. Test Period Follow-up.

7.1 Maintain observation on surviving mice for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each mouse killed each day during the test period and the five-day post-test period and the amount of liquids consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

EXHIBIT 3

PROPOSED ANTICOAGULANT RAT DRY BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill rats must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of dry bait anticoagulant rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All rats used in this test shall be Norway rats (*Rattus norvegicus*), wild caught or from a wild rat colony; or albino rats (Wistar Strain preferred). Ectoparasite control with 5% Sevin (1-naphthyl-N-methylcarbamate) dust is permissible if applied to both test and control animals not less than 7 days prior to start of test. The test rodents should fall within the following sexually mature weight classes (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weight between sexes
Laboratory rat	150	300	50
Norway rat	175	400	65

3. Pre-test Holding Conditions.

3.1 All rats used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial rat diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 25 to 25 C; Relative humidity, 50±10%; Light, twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners), 5% by weight; Corn oil, 5% by weight. Combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

4.3 When technical or concentrated toxicants are to be mixed, the baits should be formulated by adding the toxicant to the standard TSD challenge diet (4.2) when it is an acceptable carrier as prescribed on the label; otherwise follow label directions.

4.4 Technical grade toxicant normally used at 0.005% concentration should be premixed at the rate of 0.1 g of technical to 99.9 g of cornstarch using the dry dilution method. Technical grade material normally used at 0.025% can be mixed at the rate of 0.5 g of technical to 99.5 g of cornstarch, also using the dry dilution method. These newly formed concentrates can then be further mixed by combining them with the standard TSD challenge diet at the rate recommended on the label (usually 1:19). The weight of rolled oats used in the final mix is reduced by the weight of toxicant and cornstarch added in the final mix.

5. Procedure.

5.1 Ten male and ten female rats are individually caged.

5.2 Water should be available *ad libitum*.

5.3 The rodenticide-treated food and the standard TSD challenge diet are each offered in separate cups in excess of the daily food requirements. The gross weight of each cup and its contained food are determined daily and returned to the starting weight by addition or complete replacement of the given food. Each day record the quantity of each food consumed during the preceding 24 h.

5.4 The position of the bait and the standard TSD challenge diet cups in the cages are reversed every 24 h to counter any feeding position preference of the rats. The test rats have a free choice between treated and untreated food.

6. Test Period.

6.1 Maintain test period for 15 days, unless a 100% mortality of test rats is recorded prior to that time. Remove dead rats daily or as observed.

6.2 Remove toxicant at the end of the 15-day test period and leave the untreated food.

7. Test Period Follow-up.

7.1 Maintain observation on surviving rats for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each rat killed each day during the test period and the five-day post-test period and amount of treated and untreated food consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum of 33% of the food consumed had been treated with the toxicant and if a minimum mortality of 90% is obtained.

EXHIBIT 4

PROPOSED ANTICOAGULENT MOUSE DRY BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill mice must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide and Rodenticide Act, as amended. It is designed to determine effectiveness of dry bait anticoagulant rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All mice used in this test shall be house mice (*Mus musculus*), wild caught or from a wild mouse colony; or albino mice (Swiss-Webster preferred). Ectoparasite control with 5% Sevin (1-napthyl-N-methylcarbamate) dust is permissible if applied to both test animals and control animals not less than 7 days prior to testing. The test rodents should fall within the following sexually mature weight class (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes
Albino mice	15	30	5
Wild trapped and colony house mice	10	20	3

3. Pre-test Holding Conditions.

3.1 All mice used in this test method must be held for observation in the laboratory for a period of one week prior to testing. A commercial mouse diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50 ± 10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; rolled oats (steamed), 25% by weight; powdered sugar (10 x powdered confectioners), 5% by weight; corn oil, 5% by weight; combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

4.3 When technical or concentrated toxicants are to be mixed, the baits should be formulated by adding the toxicant to the standard TSD challenge diet (4.2) when it is an acceptable carrier as prescribed on the label; otherwise follow label directions.

4.4 Technical grade toxicant normally used at 0.005% concentration should be premixed at the rate of 0.1 g of technical to 99.9 g of cornstarch using the dry dilution method. Technical grade material normally used at 0.025% can be mixed at the rate of 0.5 g of technical to 99.5 g of cornstarch also using the dry dilution method. These newly formed concentrates can then be further mixed by combining them with the standard TSD challenge diet at the rate recommended on the label (usually 1:19). The weight of rolled oats used in the final mix is reduced by the weight of toxicant and cornstarch added in the final mix.

5. Procedure.

5.1 A test group consists of ten male and ten female mice, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group needs to be included.

5.2 Water should be available *ad libitum*.

5.3 The rodenticide-treated food and the standard EPA rodent laboratory diet mix are each offered in separate cups in excess of the daily food requirements. The gross weight of each cup and its contained food are determined daily and returned to the starting weight by addition or complete replacement of the given food. Each day record the quantity of each food consumed during the preceding 24 h.

5.4 The position of the bait and standard TSD challenge diet cups in the cages are reversed every 24 h to counter any feeding position preference of the mice. The test mice must have a free choice between treated and untreated food.

5.5 Provide shelter in each cage. Use empty soup or beverage cans with one end removed and slightly flattened to reduce rolling. Use one can per five mice.

6. Test Period.

6.1 Maintain test period for 15 days, unless a 100% mortality of test mice is recorded prior to that time. Remove dead mice daily or as observed.

6.2 Remove toxicant at the end of the 15-day test period and leave the untreated food.

7. Test Period Follow-up.

7.1 Maintain observation on surviving mice for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each mouse killed each day during the test period and the five-day post-test period and the amount of treated and untreated food consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum of 33% of the food consumed had been treated with the toxicant and if a minimum mortality of 90% is obtained.

EXHIBIT 5

PROPOSED ACUTE RAT LIQUID BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill rats must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of acute liquid rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All rats used in this test shall be Norway rats (*Rattus norvegicus*), wild caught or from a wild rat colony; or albino rats (Wistar Strain preferred). Ectoparasite control with 5% Sevin (1-napthyl-N-methylcarbamate) dust is permissible if applied to both test and control animals not less than 7 days prior to start of test. The test rodents should fall within the following sexually mature weight classes (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes
Laboratory rat	150	300	50
Norway rat	175	400	05

3. Pre-test Holding Conditions.

3.1 All rats used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial rat diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50±10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet mix shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners), 5% by weight; Corn oil, 5% by weight. Combine dry ingredients together, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

5. Procedure.

5.1 A test group consists of ten male and ten female rats, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group need be included.

5.2 Provide each cage with a feeder filled daily with the standard TSD challenge diet (4.2).

5.3 Provide each treated cage with equal numbers of graduated no-drip waterers. Fill half the waterers with tap water and the other half with the test liquid bait formulation diluted with tap water according to use directions. A minimum of 20 ml of each liquid, water and liquid bait, per animal per day should be available. Waterers should be supplied in sufficient quantity to exceed this demand. Replenish liquids as necessary so that both are always equally available to test animals. Reverse position of the waterers daily. The control group shall be provided with graduated no-drip waterers filled only with tap water. Each day record the quantity of each liquid consumed during the preceding 24 h.

6. Test Period.

6.1 Maintain test period for three days, unless a 100% mortality of test rats is recorded prior to that time. Remove dead rats daily or as observed.

6.2 Remove toxicant waterer at the end of the three-day test period and leave tap-water waterer.

7. Test Period Follow-up.

7.1 Maintain observation on surviving rats for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each rat killed each day during the test period and the five-day post-test period and the amount of liquids consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

EXHIBIT 6

PROPOSED ACUTE MOUSE LIQUID BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill mice must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of acute liquid rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All mice used in this test shall be house mice (*Mus musculus*), wild caught or from a wild mouse colony; or albino mice (Swiss-Webster preferred). Ectoparasite control with 5% Sevin (1-naphthyl-N-methylcarbamate) dust is permissible if applied to both test animals and control animals not less than 7 days prior to testing. The test rodents shoud fall within the following sexually mature weight class (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes
Albino mice	15	30	5
Wild trapped and colony house mice	10	20	3

3. Pre-test Holding Conditions.

3.1 All mice used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial mouse diet and water must be available to them at all times. The standard TSD challenge diet mix must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50±10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners), 5% by weight; Corn oil, 5% by weight. Combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

5. Procedure.

5.1 A test group consists of ten male and ten female mice, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group need be included.

5.2 Provide each cage with a feeder filled daily with the standard TSD challenge diet (4.2).

5.3 Provide each treated cage with two graduated no-drip waterers, one filled with tap water and the other with the test liquid bait formulation diluted with tap water according to use directions. Replenish liquids as necessary so that both are always equally available to test animals. Reverse position of waterers daily. The control group shall be provided with graduated no-drip waterers filled only with tap water. Each day record the quantity of each liquid consumed during the preceding 24 h.

5.4. Provide shelter in each cage. Use empty soup or beverage cans with one end removed and slightly flattened to reduce rolling. Use one can per five mice.

6. Test Period.

6.1 Maintain test period for three days, unless a 100% mortality of test mice is recorded prior to that time. Remove dead mice daily or as observed.

6.2 Remove toxicant waterer at the end of the three-day test period and leave tap-water waterer and feed.

7. Test Period Follow-up.

7.1. Maintain observation on surviving mice for five days following test period, reporting unnatural or unusual activities in test report. When testing arsenicals, a ten-day post-test period is recommended.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each mouse killed each day during the test period and the five-day post-test period and the amount of liquids consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

EXHIBIT 7

PROPOSED ACUTE RAT DRY TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill rats must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended. It is designed to determine effectiveness of acute dry bait rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All rats used in this test shall be Norway rats (*Rattus norvegicus*), wild caught or from a wild rat colony; or albino rats (Wistar Strain preferred). Ectoparasite control with 5% Sevin (1-napthyl-N-methylcarbamate) dust is permissible if applied to both test and control animals not less than 7 days prior to start of test. The test rodents should fall within the following sexually mature weight classes (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes
Laboratory rat	150	300	50
Norway rat	175	400	65

3. Pre-test Holding Conditions.

3.1 All rats used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial rat diet and water must be available to them at all times. The TSD rodent challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50±10%; Light, Twelve h artificial light per day, not to exceed 2153 lx at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn) 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners) 5% by weight; Corn oil, 5% by weight. Combine dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

4.3 When technical or concentrated toxicants are to be mixed, the baits should be formulated by adding the toxicant to the standard TSD challenge diet (4.2) when it is an acceptable carrier as prescribed on the label; otherwise follow label directions.

5. Procedure.

5.1 Ten male and ten female rats are individually caged.

5.2 Water should be available *ad libitum*.

5.3 The rodenticide-treated food and the standard TSD challenge diet are each offered in separate cups in excess of the daily food requirements. The gross weight of each cup and its contained food are determined daily and returned to the starting weight by addition or complete replacement of the given food. Each day record the quantity of each food consumed during the preceding 24 h.

5.4 The position of the bait and standard TSD challenge diet cups in the cages are reversed every 24 h to counter any feeding position preference of the rats. The test rats have a free choice between treated and untreated food.

6. Test Period.

6.1 Maintain test period for three days, unless a 100% mortality of test rats is recorded prior to that time. Remove dead rats daily or as observed.

6.2 Remove toxicant at the end of the three day test period and leave the untreated food.

7. Test Period Follow-up.

7.1 Maintain observation on surviving rats for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each rat killed each day during the test

period and the five-day post-test period and the amount of treated and untreated food consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

EXHIBIT 8

PROPOSED ACUTE MOUSE DRY BAIT TEST METHOD

(This method was prepared by the Technical Services Division, OPP, EPA. The format follows the style requirements of the American Society for Testing and Materials.)

1. Scope.

1.1 Products intended for use to kill mice must be tested to demonstrate rodenticidal effectiveness. This test method is applicable in connection with registration and enforcement procedures under the Federal Insecticide, Fungicide and Rodenticide Act, as amended. It is designed to determine effectiveness of acute dry bait rodenticides applied according to instructions on the labeling.

2. Test Animals.

2.1 All mice used in this test shall be house mice (*Mus musculus*), wild caught or from a wild mouse colony; or albino mice (Swiss-Webster preferred). Ectoparasite control with 5% Sevin (1-naphthyl-N-methylcarbamate) dust is permissible if applied to both test animals and control animals not less than 7 days prior to testing. The test rodents should fall within the following sexually mature weight class (in grams).

	Minimum	Maximum	Maximum acceptable difference in average weights between sexes
Albino mice	15	30	5
Wild trapped and colony house mice	10	20	5

3. Pre-test Holding Conditions.

3.1 All mice used in this test method must be held for observation in the laboratory for one week prior to testing. A commercial mouse diet and water must be available to them at all times. The standard TSD challenge diet must not be used for pre-test feeding.

4. Holding and Test Conditions.

4.1 Temperature, 20 to 25 C; Relative humidity, 50±10%; Light, Twelve h artificial light per day, not to exceed 2158 1x at cage location.

4.2 The standard TSD challenge diet shall be composed of: Cornmeal (whole yellow ground corn), 65% by weight; Rolled oats (steamed), 25% by weight; Powdered sugar (10 x powdered confectioners), 5% by weight; Corn oil, 5% by weight. Combined dry ingredients, add oil, and mix thoroughly. Assure that the mixing utensils are clean of contamination before preparing diet mix.

4.3 When technical or concentrated toxicants are to be mixed, the baits should be formulated by adding the toxicant to the standard TSD challenge diet (4.2) when it is an acceptable carrier as prescribed on the label; otherwise follow label directions.

5. Procedure.

5.1 A test group consists of ten male and ten female mice, group-caged. Include one untreated test group in each test as a control. If a series of tests are being conducted at the same time on the same species, only one untreated control group need be included.

5.2 Water should be available *ad libitum*.

5.3 The rodenticide-treated food and the standard TSD challenge diet are each offered in separate cups in excess of the daily food requirements. The gross weight of each cup and its contained food are determined daily and returned to the starting weight by addition or complete replacement of the given food. Each day record the quantity of each food consumed during the preceding 24 h.

5.4 The position of the bait and standard TSD challenge diet cups in the cages are reversed every 24 h to counter any feeding position preference of the mice. The test mice have a free choice between treated and untreated food.

5.5 Provide shelter in each cage. Use empty soup or beverage cans with one end removed and slightly flattened to reduce rolling. Use one can per five mice.

6. Test Period.

6.1 Maintain test period for three days, unless a 100% mortality of test mice is recorded prior to that time. Remove dead mice daily or as observed.

6.2 Remove toxicant at the end of the three-day test period and leave the untreated food.

7. Test Period Follow-up.

7.1 Maintain observation on surviving mice for five days following test period, reporting unnatural or unusual activities in test report.

8. Calculation and Evaluation of Results.

8.1 Maintain record of weight and sex of each mouse killed each day during the test period and the five-day post-test period and the amount of treated and untreated food consumed. Use grouped data averages for evaluation. The test product is considered satisfactory if a minimum mortality of 90% is obtained.

RODENTICIDES—FUMIGANTS

The information on the test methods or procedures for evaluating fumigants (toxic gases) for rodent control is sparse.

Laboratory Methods. It may be necessary to draw on some of the laboratory procedures used by entomologists or to rely on the methods used to evaluate euthanasia materials for laboratory animals and unwanted cats and dogs. Glen and Scott (1973) for example, describe a simple chamber for the use of carbon dioxide as a means of euhanasia for cats; Smith et al. (1973) describe an apparatus for anesthetizing small laboratory rodents which could be readily adapted to evaluate rodent fumigants under laboratory conditions.

Field Methods. A field test used to evaluate methyl bromide as a fumigant for ground squirrels (Berry, 1938) provides a basis for designing field test methods. A rather comprehensive study on the effects of various fumgants on fleas was conducted by Steward and Mackie (1938). A woodchuck control experiment was carried out by de Vos and Merrill (1957) using gas cartridges. Some early experiments with calcium cyanide on ground squirrels by Sanders (1921) provide some useful field evaluation techniques. Miller (1954) used the open hole test to evaluate a number of potential fumigants for pocket gophers. In the course of research on a method of fumigation using a rodenticide cartridge, simulated natural burrow systems were constructed and tested by Fuhr and Silver (1947).

A test design is needed which can be used to evaluate fumigants in the laboratory and which would be a suitable technique to use for all rodent species that may be controlled with fumigants. Field evaluations will differ widely depending on the species, habitat and intended use practices.

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RODENTICIDES—TRACKING POWDERS

Tracking powders came into use almost by accidents when deaths among house mice were observed in places where insecticides, such as DDT and BHC, were used. Until the ban on DDT, it was used quite extensively for house mouse control. Anticoagulant rodenticides were found effective as tracking powders for both rats and mice and, until recently, were used more in Europe than in the United States.

Little is available on methods for evaluating tracking powders. Bentley (1958) devotes a section of his bulletin to tracking powders, with reference to their evaluation. Szuber and Brodniewicz (1964) provide a drawing of their test cage and the results achieved with some anticoagulant tracking powders. More recently, Tanaka (1973) published evaluations of rodenticides as tracking powders and describes the methods utilized in his studies. Marsh (1972) discusses, in general terms, recent developments in tracking powers.

Laboratory Methods. Laboratory methods for testing tracking powders, using commensal rats and house mice, are being developed.

Field Methods. The literature is generally lacking in field studies for tracking powders; 2 articles from England, however, (Rowe, 1957; Rowe and Chudley, 1963) do provide methods for field evaluation.

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RODENTICIDES—REPELLENTS

Although few repellents are presently used in rodent control, much research was undertaken in the past. Examples of some of the test methods used in evaluating some rodent repellents are discussed; others are cited in

the literature. Since repellents encompass such a wide range of effects, a complete coverage is not easily attained and has not been attempted here.

Repellents are generally classed by their intended use, the most common being to protect growing crops, forest trees or ornamentals. Other repellents have been developed for coating seeds to protect them from rodent depredation. Further, a great deal of effort has been expanded on repellents for commensal rats and mice to minimize damage to food packages, textiles and other materials in storage and shipment and to building materials and cables. There are also chemicals which have been explored as area repellents to keep rodents from entering and/or occupying certain areas.

RODENTICIDES—REPELLENTS FOR PROTECTING LIVING PLANTS

Although repellents have been explored over many years to protect growing crops, forest trees and ornamentals from various rodents, such as the squirrel, porcupine, rabbits and hares, few test methods have been reported in the literature.

Cardinell and Hayne (1947) describe field tests for rabbit repellents using treated twigs from apple trees. The experiments were planned to take advantage of the Analysis of Variance methods of statistical comparison. Later, Hayne (1949) described both pen and field tests for protecting gardens from cottontail rabbits. Hooven (1966) provided a technique for evaluating a repellent to protect Douglas-fir trees from hares and rabbits.

Dodge et al. (1967) described an experiment for use under semi-field conditions to test deer and rabbit repellents on trees. They utilized a randomized complete block design and analyzed the data by an Analysis of Variance.

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RODENTICIDES—REPELLENTS FOR PROTECTING SEED

Laboratory Methods. Kverno and Hood (1963) published a method of screening seed protectants. Their testing period, however, was only 3 days, which may have excluded the discovery of many aversive conditioning repellents. Lindsey et al. (1974) conducted laboratory tests on the repellency of Douglas-fir seeds coated with mestranol. He expressed the amount of treated seeds consumed as a percentage of the untreated seeds eaten. Passof et al. (1974) used the percentage of treated seeds cracked open as an index of repellency for deer mice in laboratory tests; less preferred alternate food was provided to prevent starvation should the mice be highly repelled by the treated seeds. Radvanyi (1970) expressed repellency as "percent effectiveness" and calculated his efficacy using the following formula:

$$\frac{\%\text{untreated seeds destroyed} - \%\text{ treated seeds destroyed}}{\%\text{ untreated seeds destroyed}}$$

Field Methods. In field studies of conifer seed repellents, the repellency is often measured indirectly by comparing the number of seedlings produced in an area seeded with treated seeds to that of an area seeded with untreated seeds (Passof et al., 1974; Lindsey et al., 1974).

Roy (1961) used a seed spot technique to test a specific seed protectant under field conditions. The difference in the percentage of treated and untreated seed that survived was used to evaluate the results.

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RODENTICIDES—REPELLENTS FOR PROTECTING PACKAGING MATERIALS

Just preceding World War II considerable effort was exerted to develop commensal rodent repellents or deterrents for the protection of paper and cardboard packing materials. A number of laboratory test methods were developed for screening and evaluating chemicals for repellency.

Bellack and DeWitt (1949) published one test method which was used extensively in early evaluations. This test used laboratory rats housed in individual cages and offered 2 food cups: one with a standard laboratory chow; the other with the laboratory chow with the candidate repellents included. The test period was 4 days, and consumption from each cup was recorded daily. Bellack and DeWitt (1949) developed an arithmetic expression that yielded a numerical value for repellency of treated food items with their test procedure. It took into account physiological effects, random feeding and variations of animal weights. Welch et al. (1950) used the same technique and compounds.

Feeding tests became a useful procedure for rapidly screening potential repellents. Barnett and Spencer (1953) used a slight variation of the usual feeding studies by placing the deterrent below the food item, thereby attempting to measure the influence of odor alone as a repellent.

The exposure of chemically treated materials (i.e., containers, etc.) in large enclosures containing rat populations has been used by a number of researchers (DeWitt et al., 1950; Welch, 1951; Welch and Duggan, 1952; Welch, 1954).

Stolurow (1948) reported on a laboratory testing device called the Pittsburgh Obstruction Unit. It was designed for use both in fundamental research concerned with the development of methods and to study the degree of rodent destruction of barriers treated with potential repellents. The basic principles of this method have been used in simpler and less expensive designs for testing repellents on barriers (Bellack and DeWitt, 1950). In 1948, Stolurow prepared a laboratory method for the evaluation of rodent deterrents.

Analysis of the potential repellency of various packaging and structural materials is discussed by Weeks (1959). Rats are offered food on the opposite side of a barrier and are timed to determine how long it takes them to gnaw through. The time required for barriers treated with the test repellent is compared to that required for untreated control barriers. Weeks (1959) developed another repellent test which he calls the graded strip test. A strip of corrugated paperboard is placed over a row of peanuts so that the rat must gnaw through the board to get to the peanuts. The further up the strip the rat gnaws, the less the effectiveness of the repellent. Statistical evaluation was based on the method of biological assay for graded responses used by Burn et al. (1950).

Tigner and Besser (1962) devised a new quantitative method for evaluating chemicals as rodent repellents on packing materials. Tigner (1966) later reported on a series of compounds used to treat multiwall tarps and bags; qualitative method of evaluating the chemicals as potential rodent repellents was used.

A recent paper (Bull, 1972) describes an approach to evaluating attractants and the influence different odors have in inducing Norway rats to feed at given locations. In this study the effect of certain repellents was also examined.

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RODENTICIDES—REPELLENTS FOR PROTECTING WIRE AND CABLE COATINGS

Anthony and Tigner (1967) briefly describe both laboratory and field tests of a repellent used in various types of communication wire or cables. Field studies involved a number of geographical areas, which was essential because of the different rodent species involved. Also, environmental conditions associated with severe cable damage vary from region to region.

Howard (1953) conducted laboratory tests of pocket gophers gnawing electric cables. His test method confined pocket gophers to one part of a cage; a short section of cable was positioned and fastened to block the passageway to the remainder of the cage. By this means data could be obtained for treated and untreated cables and for cables with different types of covering or insulations.

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RODENTICIDES—REPRODUCTIVE INHIBITORS

A reproductive inhibitor can be defined as an agent that can cause permanent or temporary sterility in either or both sexes, or, through some other physiological aspect, reduce the number of offspring or alter the fecundity of the offspring produced.

The numerous compounds capable of inhibiting reproduction in various rodent species have diverse and complex modes of action (Jackson, 1959; Saunders, 1968), many of which do not lend themselves to standardizing either laboratory or field evaluation methods and procedures. Both Jackson and Saunders provide reviews of the capabilities of various compounds.

Because of the intensive search for oral contraceptives for humans, a large number of references are available. The literature cited is concerned with various methods of measuring the physiological effects of different compounds on inhibiting reproduction in a variety of rodent species, particularly those commonly used in the laboratory. A few representative methods and techniques are cited to illustrate what has been used.

Laboratory Methods. Histological evaluations of the effects of chemicals on cellular modifications or makeup of reproductive systems of rodents is a procedure used in some laboratories. However, histological evaluation is laborious and expensive when conducting preliminary testing or screening new compounds (Hershberger et al., 1969). Studies of the testes in treated rats are examples of this method in determining the effects of certain chemosterilants (Pate and Hays, 1968; Ericsson, 1970; Ericsson and Baker, 1970; Gibson et al., 1967).

Many researchers have used the number of pregnant females, implantations and/or the number of rats that ovulated to determine the success of several orally active estrogens and progestins (Kincl and Dorfman, 1965). Banik et al. (1969) made a statistical analysis of the data from the females with ova or implantations using the fourfold contingency test tables of Mainland and Murray (1952). The average number of ova or implantation sites in each rat in the experimental series was compared with those of the controls.

Boris et al. (1971) demonstrated a method which may have merit in establishing more precise effects of compounds on ovulation by using the pubertal ovulation in the rat for the study of anti-ovulatory compounds. Uterotrophic responses as measured by the mean uterine ratio (milligrams of uterus per gram of body weight) is a means of expressing the estrogenicity of certain potential chemosterilants (Rooks et al., 1968; Saunders and Rorig, 1964).

Efficacy of potential rodent chemosterilants can be analyzed in laboratories by using some of the following indices: Average number of days from mating to the appearance of first litters; average total offspring produced per female; average number of litters produced per female (Generoso et al., 1971; Howard and Marsh, 1969). Survival of the offspring and their reproductive capabilities may also be used to determine efficacy expressed in the F_1 generation (Kincl et al., 1965; Clancy and Edgren, 1968). These are not the only criteria for efficacy, but are some of those commonly used.

Where a compound may affect spermatogenesis, efficacy of that compound may be determined by using a serial mating method. This technique involves placing the treated male with 1 or 2 females for a prescribed number of days and then moving him on to new females for a similar period. Thus, by pairing the males with a series of females, it is possible to determine the time period in which sterility occurred and its duration (Hershberger et al., 1969).

Field Tests (Simulated). Tests of a rodent chemosterilant may be conducted in large pens where small populations can be maintained and the tests can be replicated (Kendle et al., 1973; Gwynn, 1972a). Studies should be replicated a number of times under conditions where evaluations of effects can be quite exacting.

Field Methods. All the methods cited have value, although some may be more applicable than others, depending on the characteristics of the compound being tested, the target species and on how the material will ultimately be used in the field.

Marsh and Howard (1969) evaluated mestrano as a reproductive inhibitor in Norway rats in garbage dumps. Two dumps were treated and a third served as a control. The age structure of the rat population and population numbers were the criteria used to determine efficacy. Bowerman and Brooks (1971) carried out pen and field efficacy studies on Norway rats using quinestrol. Gwynn (1972a, 1972b) published pen and field results using still a different rodent chemosterilant.

These represent a portion of the field studies which have been conducted. Field evaluations differ slightly from one another to meet the existing situations and the intended uses of the test compounds.

Significant reproduction inhibition assumes that comparable untreated populations are used as controls to permit a sound statistical analysis. The Chi square analysis of the two-way Analysis of Variance might be the appropriate measure or the significant differences between the treated and control groups. Efficacy tests should be replicated to adequately demonstrate a high degree of reproducible results under various conditions of use. Potential chemosterilants also should be tested in widely separated geographical areas. Because evidence exists that many potential chemosterilants can be quite specific in their effects (Skinner, 1968), all species on which the product is to be used must be tested both in the laboratory and in the field.

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CANDID AND OTHER CARNIVORE TOXICANTS, REPELLENTS, REPRODUCTIVE INHIBITORS AND DEVICES

Research in predator damage control has only recently attempted to provide information on the extent of predation and possible alternative solutions to past and present control methods. Balser (1974a, 1974b) has reviewed the status of coyote control, data on livestock losses and research related to control methodology. His review and others (Cummings, 1966; Howard, 1974; Knowlton, 1972) have pointed to the paucity of data necessary for predator management.

Methods with no undesirable characteristics have not yet been developed. The belief that one method is no better or worse than another is not adequate cause, however, to either reject or justify all control techniques. Depredation control cannot be deferred until ideal methods are identified.

Adverse environmental effects from professionally-employed controls have not been demonstrated (USDI, May 1971; Berryman, 1971), and, conversely, the need for selective professional use of lethal chemicals in predator damage control has been documented (USDI, May 1971; January 25, 1974; May 30, 1974). Experienced professionals recognize the limitations imposed on various mechanical methods by rough terrain, heavy vegetation, adverse weather and other factors. Approval by the Environmental Protection

Agency of Experimental Use Permits to several western States and to the Department of Interior in 1974 for use of toxic chemicals and devices in predator control also acknowledges damage problems that have not been solved by mechanical and nonlethal means.

Changing land-use patterns and land development have affected the habitats and food choices available to both predator and prey animals. Consequently, maintenance of wild animal populations above levels sustained by available natural foods encourages or requires inclusion of domestic products in the diet. Control of animal damage to protect habitats, other wild species and domestic products is basic to sound management and includes regulation of game and non-game populations. It is an essential element in carnivore management and must be considered in the light of current and developing land-use patterns.

Among the larger carnivores that cause damage and resulting management problems are the families Canidae, Felidae and Ursidae. Bobcats (*Lynx rufus*) may prey extensively at times on domestic animals and lynx (*Lynx canadensis*) may occasionally cause damage, but offending animals usually can be managed by mechanical methods. Bears (*Ursus spp.*) and mountain lions (*Felis concolor*) may cause extensive damage at times but are typically considered game animals. Again, offending animals are managed by mechanical methods sanctioned by State wildlife agencies.

The carnivores of primary interest here are gray foxes (*Urocyon cinereoargenteus*), red foxes (*Vulpes fulva*), coyotes (*Canis latrans*), wolves (*Canis lupus* and *C. niger*), feral dogs (*Canis familiaris*) and various hybrids. Those causing most damage problems and concern at present are red foxes, coyotes, feral dogs and coyote-dog hybrids. They are particularly difficult to manage because of their inherent caution, adaptability and high reproductive rates.

Evaluation of the efficacy of various control methods is extremely difficult because of the wide variation in circumstances where controls are applied. Heavily populated areas with small farms present a markedly different environment than open range with large land holdings and few humans. Climate and weather, habitat types, predator populations and reproductive rates, food bases and feeding habits are some of the variables that often prevent replication of experiments under similar conditions. Another major impediment is the lack of adequate census techniques to measure populations and the changes induced by natural and applied controls.

Development of efficacy criteria for control methods will require cognizance of time and cost factors caused by the complex nature of predator-prey ecology and the extreme difficulty in securing uniform replicate field tests. Several years of study over extensive geographic areas under widely-varied climatic conditions will be needed to provide even minimal knowledge of predator-prey economics, behavior and ecology. A review of information and data from field studies and other sources, such as consensus standards under development by the American Society for Testing and Materials, may provide adequate guidelines for protection of nontarget species and the environment. But subjective evaluation will continue to be necessary in predator research and management until much more knowledge is available.

To summarize, effective candid control methods which meet all desired criteria do not exist. Known methods applied to minimize undesirable effects may provide efficacy data and essential damage control. Effective and useful methods vary with time and circumstance and may require removal of specific offending animals or population reduction to achieve adequate depredation control.

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CANID AND OTHER CARNIVORE TOXICANTS

Laboratory methods for evaluating the toxicology and physiology of candidate chemicals are well established and will not be considered here except as related to mode of application. Consensus standards for use now under development by the American Society for Testing and Materials may provide additional guidance in evaluation of efficacy.

Atzert (1971), Crabtree (1962), Robinson (1970), Ward and Garlough (1936), and Ward and Spencer (1947) have summarized toxicology and use patterns for historically used toxic chemicals. Field research data on the use of toxic chemicals to control canid species is limited. Of 92 research projects reported by Knowlton (1973), none included research in this area. Experimental efficacy studies of the M–44 (sodium cyanides spring-loaded ejector mechanism) are now being developed in several western States to evaluate its use in management of red foxes and coyotes.

No specific complete test protocols exist for field evaluation of toxic chemicals for predator management. Survey research data related to domestic livestock losses and relative effects of toxic baiting on coyote populations have been reported by Robinson (1948, 1953a, 1953b, 1961) and by Linhart and Robinson (1972). Effects on target and nontarget species populations are evaluated from trap-catch data and observations. Suggested methods for baiting to reduce hazards to nontarget species are given. Data recorded indicate that reduced coyote populations were followed by increased populations of nontarget carnivore species. Continued use of additional control methods in the survey areas prevents isolation of specific effects of each method. The scope of the reports do provide extremely useful information on the effects of control and animal population trends over a time span.

Arrington and Edwards (1951) employed antelope population surveys to evaluate effectiveness of predator reduction on antelope productivity. Toxic chemicals were the primary reduction method, but overlapping use of more than one chemical and other methods prevented isolation of the effects of each method. Coyote population reduction was reported to result in greatly increased antelope productivity; however, coyote populations were not indexed.

Beasom (1973a, b) discusses relative effectiveness and selectivity of various methods of predator reduction to enhance wild turkey and deer productivity. High selectivity by the M–44 was noted.

Balser, et al. (1968) reported results of a well-designed field study of predator reduc-

tion and waterfowl nesting success. Several reduction methods were used. Selectivity is not thoroughly discussed, but a similar design would be useful in evaluating specific methods if experimental areas were adequate.

Field tests of baits must consider food preference and availability to target species. Bait acceptance by extremely wary individuals or species usually requires that the chemical either be masked or be relatively tasteless. Also seasons, weather, temperature and other factors affect bait acceptance.

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CANID AND OTHER CARNIVORE REPELLENTS

Few studies of potential repellents tested on wild carnivores have been reported. Definitive results from laboratory and pen tests are difficult to obtain and are not necessarily representative of behavior in wild free-ranging species under field conditions. In addition, highly variable physical and biological factors may obscure the real effects of potential repellents. Preliminary screening of methods with domestic animals may be useful before evaluation with wild species and field tests if such limitations are recognized.

Nesse (1971) and Swanson and Scott (1973) reported results of limited field trials of the "Gerhardi Livestock Protector" to protect goats and sheep from coyote depredation, but replications were not adequate for accurate evaluation. Pfost (1971) conducted field trials of the Gerhardi device to protect weeder geese from dogs and coyotes. Replications were not uniform, but the study was reasonably well designed.

Gustavson et al. (1974) attempted to induce conditioned aversion in coyotes with lithium chloride; the discussion is not adequate for thorough evaluation. Jankovsky et al. (1974) reported briefly on the results of coyote repellent research in Colorado. Again, discussion of methodology and results is incomplete, and no wildlife census techniques were employed.

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CANID AND OTHER CARNIVORE REPRODUCTIVE INHIBITORS

Few studies have been directed toward management of wild canids by controlling reproduction. A wealth of information has been accumulated from screening tests of potential human contraceptives. These studies and others carried out with rodents, logomorphs, dogs and cats provide a reference source. Preliminary testing on domestic species to clarify physiological effects may be necessary because of difficulty in securing normal reproductive cycles in captive wild species. Pen tests with target species are essential, since separation of physiological effects from those caused by inadequate application techniques and other factors is extremely difficult with highly mobile wild species under field conditions.

Branker (1943) and Jackson (1953) have demonstrated clinical use of diethylstilbestrol (DES) to manage canine mismating. Kennelly (1969) determined abortifacient effects of mestranol in dogs during early gestation. Modification of Kennelly's procedure to determine effects at specific stages in the reproductive cycle is feasible. Balser (1964b) reported abortion in captive coyotes induced by a single oral dose of DES.

Modification of test designs employed by Linhart and Enders (1964) and Storm and Sanderson (1969) to determine reproductive inhibition in captive female foxes is necessary to delineate specific stages of reproduction affected. Additional changes are needed to evaluate chemical effects on spermatogenesis and male fox fertility.

Linhart (1964) reported results of field tests to determine bait acceptance by wild foxes, but the test design does not provide population measurements. Oleyar and McGinnes (1974) conducted field trials of DES to reduce fox reproduction.

Balser (1964b) discussed results of field trials with DES to reduce coyote reproduction. Linhart et al. (1968) reported results of the field trial by Balser (1964) and several others to determine effects of DES on reproduction in wild coyote populations. Inclusion of several trials with the variation in methodology employed prohibits a thorough evaluation. The authors suggest modifications of techniques and application but do not recommend specific methods.

Improved techniques for application of reproductive inhibitors and methods to measure populations are needed. In many wild species, relative indices may be the only feasible method to determine population levels and changes.

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CANID AND OTHER CARNIVORE CONTROL DEVICES

Published research reports on control devices are few. Extensive overlap with toxic chemicals occurs since chemicals are employed in mechanical devices. Devices are also often used simultaneously with other methods. Separation of effects, therefore, is difficult.

Beasom (1973a, b) presents data and discusses the use of the M–44, shooting, steel traps and strychnine baits to remove predators and enhance productivity of wild turkeys and white-tailed deer in specific study areas. Simultaneous use of several methods reduces the value of the data for determining effectiveness and selectivity of the various methods. However, high selectivity was noted for the M–44 and shooting when compared to traps and baits. Predator track counts and rodent snap trap transects were utilized to establish relative population indices, but specific census techniques are not provided.

Robinson (1943) discusses the use of steel traps and the "Humane Coyote Getter" for predator control. The report compares efficiency and selectivity, seasonal and weather effects, age of animals removed, experimental areas, and defects of the methods. Census techniques are not provided except indirectly by indices based on trap catch data. Hey (1964) provides a limited amount of data regarding use and selectivity of the "Humane Coyote Getter" in comparison with other control methods and discusses hazards to nontarget species. No census or index methods are discussed.

Robinson (1953, 1961) and Linhart and Robinson (1972) provide data indicated selectivity of steel traps in relation to species populations and discuss population trends of target and nontarget species in predator control areas. Population indices are taken from trap-catch data. These reports do not provide test protocols, but they include highly useful information for developing test methodology.

Field methods reported by Balser et al. (1968) require adequate experimental area and population indices.

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DOMESTIC DOG AND CAT REPELLENTS AND REPRODUCTIVE INHIBITORS

The number of protocols in the literature for tests of repellency with dogs and cats are limited. A comprehensive search would require consultation with the workers in this field.

Repellents. Huebner and Morton (1964) published a tentative test of efficacy of 5 commercially available dog repellents. Papendeck (1968) conducted laboratory research on capsaicin by spraying the test dogs' muzzles and eyes. The agent produced an aversive response in dogs in the laboratory. Histological examinations showed that the spray did not cause lasting damage to the eyes.

Reproductive Inhibitors. Balser (1964) specified the attributes of an ideal reproductive inhibitor. It must be effective in a single oral dose, offer a wide margin of safety between the effective dose (ED) and the lethal dose (LD), remain chemically stable at an ED of less than 500 mg, be relatively tasteless and odorless, and have a sterility effect for one breeding season. The author's protocol was tried with the synthetic estrogen diethylstilbestrol.

Yasmuth, Rowe, Doege and Bangxang (1970) tested ovarian suppressants for dogs. They subcutaneously administered 2 ml medroxyprogesterone acetate to 60 free-roaming females. Blood tests, counts of mortality and frequency of litters were taken. All females had at least 1 litter before treatment. Observations were made for a year.

Lunnen, et al. (1974) published a laboratory protocol for inducing impotency in male dogs. Male beagles were immunized with a purified bovine lutenizing hormone. The dogs were sacrificed at the end of the experiment. The testes, epididymides and prostate, adrenal, thyroid and anterior pituitary glands were excised and measured for weight differences.

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SKUNK TOXICANTS, REPELLENTS AND REPRODUCTIVE INHIBITORS

Skunks are generally beneficial because they eat insects, slugs, mice and a variety of fruits and berries. In most States, they are listed either as game animals or fur-bearers and cannot be shot, trapped or poisoned without permission from the State game and fish department.

The following skunks occur in the U.S. (Burt and Grossenheider, 1952; Hall and Kelson, 1959): the common striped skunk (*Mephitis mephitis*) is abundant throughout the U.S. and most of Mexico and Canada; the hooded skunk (*Mephitis macroura*) is found in Central America, Mexico and the Mexican border of southeastern Arizona; the hognose skunk (*Conepatus leuconotus*) occurs in Mexico and the Southwest; and the spotted skunk (*Spilogale putorius*) is found over most of the U.S. except the northeastern States.

Toxicants. Lynch (1972) tested kill-efficacy on a large waterfowl marsh divided into 3 areas (a treatment area, a control area and an untreated buffer area between). In the control area he put out simulated duck nest sites with 4 untreated eggs in each nest. In the treatment area each nest site contained 4 treated eggs.

Seyler and Niemeyer (1974) used strychnine-injected eggs for emergency control of rabies in skunks in Montana. They put out scent post lines to determine the population densities of carnivores. They also evaluated the effects on nontarget species.

Repellents. There is little specific literature on repelling or controlling skunks. Scientific methodology of testing has only been worked out for those species where monetary losses, for example, to forestry or agriculture are high.

Reproductive Inhibitors. Some preliminary work on reproductive inhibitors for both skunks and raccoons has been reported (Nelson and Linder, 1972).

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WHITE-TAILED DEER REPELLENTS

Repellents are now widely used in the U.S. In general, 2 types of repellents have been used for deer, the odor repellents and the taste repellents.

There are several methods for testing the efficacy of repellents on vegetation in relation to deer damage. The oldest method has been to lay out line transects and establish plots in treated and untreated areas (Besser and Welch, 1959). The most widely used method (Duncan and Whitaker, 1959), however, is to survey transects in the field on treated and untreated areas. These methods require a long period of time and the results are difficult to evaluate.

Another method involves pen tests where materials are treated and then presented to the test animals. An additional modification consists of providing pens with captive animals and planted blocks of treated and untreated seedlings. These pen tests yield more controlled data. In these experiments, the seedlings are planted in 2½- and 1-acre enclosures holding deer and hares. A control and a repellent planting are used to evaluate the test chemical. Dodge, Loveless, and Kverno (1967) modified the techniques for the planted enclosures. These tests were of randomized complete block design in which 10 blocks with 10 trees per block for each of the treatments were used. The untreated control pen must be observed routinely until 60 to 80 percent of the reference seedlings sustain animal damage.

Campbell and Bullard (1972) devised a preference test design using an apparatus which presents 2 food samples. The arrangement can be quickly changed to offer 2 more small dishes of a test material. These presentations are made so that at least 2 completely different sets of experiments can be executed at one time. Four to 10 deer are routinely tested once or twice daily for 1 or 2 weeks. The test consists of at least 10 to as high as 30 choices. Normally a deer can complete 20 choices in about 6 minutes. The animals are tested against a repellent standard. One objective of the test is to obtain marginal acceptance.

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FIELD CENSUS TECHNIQUES

Population censusing under field situations is essential to adequately evaluate many vertebrate pest control agents. Some means must be used in determining the number of animals before treatment and again at post-treatment to establish efficacy. Censusing techniques are fundamental to all population studies designed to measure the influence of chemicals on the target or nontarget populations.

Censusing techniques for the orders Rodentia and Lagomorpha are numerous and varied. Probably no technique is perfect; each has its strengths and weaknesses. The researcher must make every effort not only to determine which methods of population estimation best suit his purposes but also to apprise himself of all the weaknesses of his chosen methods. This enables the best interpretation of the results within the framework of his outlined test methods or procedures. In a general vein, Murray (1957) warns of the danger of error in most censusing techniques due to the selective distribution of animals according to habitat type within an area being censused. Censusing methods which in themselves affect the population (for example, fatal trapping, shooting, etc.) should not be used unless statistical procedures can be applied to correct for these effects. When practical, more than one censusing procedure should be used.

Many comparisons of techniques are available. Edwards and Eberhardt (1963) tested various methods of estimating a population of cottontail rabbits (*Sylvilagus floridanus*) from live-trapping data against a known population. They suggested, with some qualifications, that linear regression or maximum likelihood estimation (MLE) methods were the most useful. When several procedures were tried on grey squirrels (*Sciurus carolinensis*) in Maryland, Flyger (1959) found that a Schnabel (1938) population estimate, based on what Flyger termed "trap-sight records," was best for estimating the population. He used visual sightings because he felt that it lessened bias injected by trap-shy or trap-prone animals. The Schnabel method is a variation of the Lincoln Index that uses the average of a series of ratios rather than one and is considered, therefore, to be less prone to sampling error. Nixon et al. (1967) also felt that population estimates of squirrels (*S. carolinensis* and *S. niger*) from the Schnabel method were low because of capture frequency variation for different individuals. They preferred the simplified MLE method (Edwards and Eberhardt, 1967).

Robinette et al. (1974) conducted tests of 10 different types of strip censuses. The 3 most satisfactory methods were those of Kelker (1945), Anderson and Pospahala (1970) and King. King's method was described by Leopold (1933) in his book on game management. Hayne (1949) presents a critical evaluation of the King method.

Beaver census techniques are reviewed and evaluated in light of an extensive census operation by Hay (1958). After checking several census methods, he found aerial counting of food caches to be the most practical and reliable.

Forest rodent censusing to determine conifer seed depredation potential provides an interesting example of a situation where the techniques used are not aimed toward absolute enumeration, but toward population indices for use in evaluating rodent control operations. Lines of live or snap traps are often used for these determinations. However, some researchers feel that the "seed-spot" census method is more useful to foresters than trapping because of its relative ease in use (Tevis, 1956; Moore, 1949–50). Tevis discusses the method thoroughly.

It is based on the assumption that the percentage of piles of seeds placed in an area that are disturbed is proportional to the number of seed eaters in that area. Moore gives a key for determining what species disturbed each seed spot.

Emlen et al. (1949) reviewed Norway rat (*Rattus norvegicus*) census methods in some detail and discussed the shortcomings of some of the various methods. Chitty (1942) and Bentley et al. (1957) used wheat consumption as a poulation index for Norway rats in sewers. Rohe (1966) gives guidelines for the censusing of roof rats (*Rattus rattus*); he used snap-traps and nontoxic paraffin-grain bait blocks to test for the presence of rats and their relative numbers. Nelson and Clark (1973) published a method for correcting trapping results by taking into account sprung traps in calculating catch effort. Emlen et al. (1957) and Tester and Emlen (1960) tested dropping boards as a method of obtaining population indices.

Census methods for pocket gophers (*Geomys bursarius*) and moles (*Talpa europa Linnaeus*) are presented by Beck and Hansen (1966) and Mead-Briggs and Woods mating pocket gophers by counting new signs was also reported by Reid et al. (1966).

When faced with the task of censusing a population, 2 sources for background information are Dice (1941) and Davis (1956). Dice reviews many of the methods for dealing with mammalian populations, and Davis gives an outline of field and laboratory methods for accumulating and analyzing data on rodent populations. In addition to these sources, Chitty and Shorten (1946) present a compilation on the techniques for the study of Norway rats. And, finally, Tepper (1967) compiled a bibliography entitled, "Statistical Methods in Using Mark-Recapture Data for Population Estimations."

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PART VI

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PART VI.—ENVIRONMENTAL CHEMISTRY

INTRODUCTION

The suggested test methods or "protocols" described here were developed from extensive literature searches, workshops and consultations with academic, governmental and industrial experts in environmental chemistry of pesticides. Note that the protocols currently included do not address the fate of pesticides in aquatic or runoff sediments.

The protocols described are considered acceptable for compiling registration data at this time. The remaining discussion in this introduction provides interpretative information on certain environmental chemistry terminology and/or techniques included in the protocols.

The dissipation of pesticides in the environment occurs by various biological, chemical and physical processes, and in general, the test methods described here are designed to characterize the potential environmental significance of these various processes. For purposes of registration, the primary objective of laboratory and field soil dissipation studies should be the characterization of the *rate* and *pathways* (products) of dissipation as opposed to *mechanisms* (microbial versus chemical, etc.). Accordingly, in discussing test methods, the term "dissipation" is used, except where more specific terminology is justified.

Since knowledge of all major products formed is essential, studies with radiolabeled pesticides is necessary. As indicated in the EPA Guidelines for Registering Pesticides, "radiolabeling should be in a strategic portion of the molecule that will remain in the significant degradation products." Usually, but not always, such labeling will involve carbon 14 in an aromatic ring. However, some highly complex pesticides may require radiolabeling at several points in the molecule or with dissimilar isotopes to assure adequate coverage of all chemical transformations. To avoid undue emphasis on the use of any one isotope in preference to another, the more general terms "radiolabel," or "radioactivity" are used in descriptions of suggested methods.

In assembling test methods and protocols, 2 levels of product identification were assumed: Characterization and complete chemical identification. It is important that the material balance be studied during metabolic or photochemical studies, since the quantitative significance of products may then be assessed. Efforts should be made to identify or characterize products that do not appear to be of a transitory nature.

Complete chemical identification implies rigorous and complete establishment of chemical structure and spatial conformation of a molecule by comparison of chemical and physical properties (for example, mixed melting point, spectral measurements, etc.) with those recorded for an authentic specimen. If authentic material is not available, the structure may be postulated on the evidence of instrumental determinations and chemical reactions. The criteria for identification should conform to the standards accepted by reputable chemistry journals. Specific methods for quantitative analysis may be devised after a substance has been completely identified.

Product identification should be conducted on all materials comprising more than 10 percent of the initial application, or 0.1 ppm, whichever is greater at the time of sample analysis. Other products in excess of 0.01 ppm should be identified where feasible. Where such chemical identification is not feasible, attempts should be made to characterize the product. Characterization of unidentifiable products may include such factors as TLC mobility, GC retention time, relative polarity, UV absorbance on TLC (+ or −), radioactive content (percent of original or total recovered), or possible TLC spray reactions.

It is recognized that polar, polymeric, and bound materials present a major problem. In most cases their identity cannot readily be established and their homogeneity is difficult to determine. Their quantitative significance and homogeneity should be investigated through studies of material balance. In the case of bound materials, their distribution between substrate fractions should be examined. (For example, association with certain fractions of soil or tissue may be investigated by classical fractionation techniques.) Chemical studies may not reveal the structure of such compounds, but data may be obtained to support their characterization. Although data, such as ultraviolet spectra, infrared spectra, etc., may not be readily subject to interpretation, the data can form reference points for comparison, confirmation and repetition of experimental studies.

Investigations should be sufficiently thorough to support the characterization of the material in question. For example, the characterization of a photolysis product as "polar" should be based on broader evidence than the fact that it remains immobile in the solvent system chosen for chromatography of the major products. At the termination of a photochemical experiment, for example, data should indicate the relative stability of a compound to light in solution and in the solid states. The structures of the major photolytic products should be characterized, together with their quantitative significance, at several time intervals after the initiation of the study.

Extrapolation of the results of laboratory studies to environmental behavior can be undertaken only with the realization that many pitfalls are involved and that few experiments have been performed to show the failure or success of such attempts.

CHEMODYNAMIC PARAMETERS—WATER SOLUBILITY

The classical definition of a solution is "a homogenous mixture constituting one phase only, of two or more components" (*1*). The application of this definition is clear if a substance forms a true solution in which the particles of solute are of molecular dimensions, for example, sucrose or urea. A suspension or an emulsion, by contrast, contains particles visible to the naked eye or microscope lens. Between these two extremes, however, exist colloidal systems formed by substances of low solubility. The determination of solubility of pesticides may often be complicated by the problem of formation of colloidal systems, since many pesticides are soluble to less than 100 ppm in water (*2*).

Recommended Procedures. It is important that the material under study be as pure as possible, especially if the method of solubility determination involves the analysis of a saturated solution. The method of analysis should be as specific as possible. The determination of radioactivity in a solution of a compound previously crystallized to constant specific activity is one example of a technique for determination of solubility which would satisfy this criterion.

Classical Method:—The classical method (*2, 3*) requires equilibration at the recommended temperature followed by determination of the concentration of material in solution. Use of radioactive material facilitates this measurement. The separation of the saturated solution from the dispersed particles can be achieved by centrifugation. It is recommended that: (1) The centrifugation be carried out at 12,000 g (with temperature control) until there is no change in concentration of the supernatant liquid; and (2) the solubility in water be determined at 3 temperatures—15°, 25° and 35° C.

The effect of pH on the solubility should be studied if the pesticide is acidic or basic, and the pH range studied should encompass the pK_a or pK_b values (*4*).

The work by Gunther et al. (*5*) serves as a primary reference to solubility of pesticides. Not only are solubility values given with references, but also methods are described and examples are given. However, some details of technique are either omitted or bear re-emphasis. The following procedure should be followed, in general:

1. Interference of soluble impurities can be minimized by simple expedient of preparing several successive saturated solutions from the same sample of chemical. Solubility values should not change.

2. Some caution should be used in applying filtration to saturated solutions from very slightly soluble chemicals. Adsorption from such a dilute solution may be significant.

3. A recommended technique is to prepare saturated solutions both by shaking the chemical with water at room temperature and by preparing a supersaturated solution (normally at elevated temperature), which is also equilibrated by shaking at room temperature. Both approaches should yield the same solubility value.

Nephelometric Method. The nephelometric method is a rapid method that may be useful for many pesticides especially if radioactively labeled material is not available. The fact that it is rapid not only makes it economically advantageous but also makes it applicable to compounds that decompose slowly in water. An example is maneb, which, because of slow decomposition, releases an ever increasing amount of manganese as it is shaken with watetr. Davis and Parke (*6*) have described a nephelometric method. This method is recommended for compounds possessing solubility greater than 1 ppm and gives only an approximate value. To obtain the true solubility value, another method should be employed.

The method requires a turbidimeter, such as the Hach Turbidimeter 2100, and an ultrasonifier, such as the Biosonic IV. A 1 percent solution of the chemical in acetone or ethanol is used to obtain an initial rough estimate of solubility by observing the presence or absence of turbidity with injection of various amounts of solution into approximately 30 ml water and sonication for 10 sec by a sonic probe. If the suspensions are unstable and quickly coalesce into larger particles, the method will not work for the particular chemical. It is not generally fruitful to search for a stabilizing agent but occasionally gelatin, methocel, etc., may help stabilize the suspension.

If the suspension is stable, a series of suspensions are prepared and sonicated. The sonication is timed by a stop watch and the temperature measured just after the turbidity of the sample is measured. The turbidities plotted against the total concentration should give a straight line. Other concentrations are prepared, as necessary, to define the line and also the "zero" turbidity caused by the water used and by the "insoluble impurities in the chemical sample. In most cases the solubility (point of "zero" turbidity) can be estimated by determining a best straight line either by eye or linear regression. Solubility at other than room temperature can be achieved by use of constant temperature water baths.

Method for Hydrophobic Chemicals. The water solubility of hydrophobic chemicals, such as DDT, where the values are in the range of parts per billion is difficult to measure. Such chemicals take much longer time to reach equilibrium. The following procedure should be adopted (*7*).

Before starting the experiment, it should be determined that the water contains no organic impurity. Such an impurity may be removed by extracting it with an organic solvent then boiling or passing the water through a column containing a suitable resin. The compound under investigation should be dissolved in ether and deposited as a thin layer on the wall of the container (either a large glass container or a carboy). The ether is evaporated and water added.

The system should be stirred slowly and the water analyzed for the concentration of the chemical as a function of time, until a constant value is reached. At this point the stirring should be discontinued and the water analyzed again for the concentration of the chemical. Stirring may cause the formation of aggregates; when the stirring is discontinued, such aggregates may settle down and the concentration of the chemical in the water may decrease. Therefore, additional samples should be analyzed for the concentration of the chemical until there is no further change. This would represent the true solubility of the compound. The analysis of the compound may be carried out using radioactive, gas-liquid chromatography or any other suitable technique.

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CHEMODYNAMIC PARAMETERS—PARTITION COEFFICIENT

Once a pesticide finds its way into a living system, its accumulation and transport, to a great extent, will be governed by its partition characteristics—water solubility, polarity of the molecule, affinity of the chemical towards fats and the nature of the individual biological polymer. The well-known Overton-Meyer hypothesis indicated that lipid solubility of a toxic chemical may be related to its toxicity (*1*). It is now generally accepted that the partition coefficient (defined as the ratio of concentration of the chemical at equilibrium in octanol and water phase) may be related to toxicity and electronic parameters of a compound (*2*). This concept has found considerable application in the development of new drugs. Since pesticides generally possess low water solubility and high lipid affinity, the octanol/water partition coefficient data may provide much information about their distribution, accumulation and persistence in a living system (*3*). Such data also may be helpful in planning toxicological and monitoring studies.

Method. The octanol/water partition coefficient may be determined by the general method described below.

Experiments should be carried out by equilibrating the pesticide in n-octanol/water mixture. Pesticides and n-octanol of the purest grade available should be used; the octanol should be distilled. Since octanol has a finite solubility in water, both water and octanol should be equilibrated with each other. The pesticide should be dissolved in n-octanol and equilibrated against water. The system should be shaken at constant temperature, centrifuged and the concentration of the pesticide in the n-octanol and water phase should be determined by a suitable analytical technique. The time required for equilibration and the ratio of octanol to water will depend on solubility of the compound and will be determined by a trial-and-error method. The partition coefficient is calculated from the ratio of the concentration of pesticide in n-octanol and water, after equilibrium. Experiments should be carried out at 2 different concentrations of a pesticide.

Interpretation of Results. If a pesticide has a high partition coefficient, it is likely that it will bioaccumulate or be persistent. If the partition coefficient is greater than 100, a fish bioconcentration experiment may be indicated. If, on the other hand, the partition coefficient is less than 10, bioconcentration is unlikely.

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CHEMODYNAMIC PROPERTIES—DISSOCIATION CONSTANTS

The dissociation constants of acidic and basic pesticides in water are essential to understanding their environmental behavior. The relative distribution of undissociated, cationic or anionic species of a pesticide can affect its availability in such processes as oxidation-reduction reactions, ion exchange, coagulation, adsorption and leaching (*1, 2*).

Numerous methods for determining dissociation constants have been used and are briefly reviewed by Nelson and Faust (*1*). The usual method is to conduct a potentiometric titration of the chemical with strong acid or base as required (*1*); however, potentiostatic (*3*) or spectrophotometric (*4*) methods might also prove useful.

METHODOLOGY

Materials. (1) The pesticide should be analytical reference grade. (2) For titrants, standardized 0.10 *N* NaOH is prepared and the sodium carbonate and carbon dioxide are eliminated by conventional techniques in addition standardized 0.10 *N* HCl is also prepared. (3) For ionic strength control, sodium perchlorate or potassium chloride may be useful.

Equipment and Procedure. An expanded scale pH meter is used with glass and saturated calomel electrodes. The instrument should be standardized and linearized against standard buffer solutions. The temperature should be monitored constantly and maintained within 25° ±0.1°C. Titrations are performed under an atmosphere of nitrogen that is run through sodasorb and distilled water. This technique prevents evaporation of the titrant solution.

The steps below provide the necessary data for calculating the acidic dissociation constants. The determination of basic dissociation constants will correspond with the substitution of HCl for NaOH.

Plot *pH* versus volume (ml) of NaOH to yield the typical S-shaped titration curve for a monobasic acid. If the acid is polybasic, there may be more than one inflection point.

Plot the Δ pH/Δml versus average volume (*ml*) of titrant to yield the first derivative curve which, in turn, reveals the equivalence point or point of inflection of the titration curve.

In the S-shaped curve, choose a point above halfway to the equivalence point, where a well-buffered region exists. In this region, the concentration of the undissociated acid is approximately equal to the concentration of the anion, and the *pH* change is small with each addition of NaOH. Consequently, there is less chance for error in the *pH* reading. The chosen point yields values for $[H_3O^+]$, V, and v for insertion into the equation:

$$K=\frac{[H_3O^+]\left([H_3O^+]+\frac{Mv}{V}\right)}{\frac{M}{V}(V_{e-v})-[H_3O^+]} \qquad (1)$$

where
$[H_3O^+]$=hydronium ion concentration.
K=classical or concentration dissociation constant.
M=normality of NaOH.
v=volume of titrant added, i.e., the buret reading.
V=total volume.
V_e=theoretical volume of titrant added at the equivalence point, calculated from the weight of acid pesticide used.

This equation yields the classical or concentration acidic dissociation constant, which varies with ionic strength. If the acid under investigation is polybasic or the dissociation constants are close together, then the equation cited above should be modified accordingly. If it is assumed that activity coefficient equals 1, then $(H_3O^+)=[H_3O^+]$ and $pH=-\log[H_3O^+]$. Equation 1 is a straightforward derivation from the law of electoneutrality and appropriate substitutions. The $[OH^-]$ curve was assumed to be small and negligible in the portion of the titration curve from which the calculation of K was made.

A theoretical titration error at the equivalence point can be calculated from:

$$\frac{V_e-V_q}{V_q}\times 100=\text{percent titration error} \qquad (2)$$

where V_e=actual volume of titrant added at the equivalence point.

Classical K values as calculated from Equation 1 by Nelson and Faust (*1*) are listed in Table 1 for several ionic strength values. A comparison of the values with those reported in the literature by other investigators is presented in Table 2. For more accurate measurements of pK_a and pK_b, appropriate corrections for the influence of ionic strength may be necessary (Table 1).

TABLE 1.—*Acidic dissociation constants* (K) *at various ionic strengths* (I)

[From: reference 1]

Compound	I	K	Theoretical titration error (percent)
Dalapon	0.00	1.25×10^{-3}	−1.15
	0.05	1.45×10^{-3}	
2,4-D	0.01	1.7×10^{-3}	+0.34
	0.05	1.86×10^{-3}	
2,4,5-T	0.012	1.29×10^{-3}	+0.47
	0.03	1.31×10^{-3}	
2-CPA	0.02	1.04×10^{-3}	−0.86
	0.05	1.20×10^{-3}	
4-CPA	0.01	1.6×10^{-3}	−1.13
	0.05	1.12×10^{-3}	
MCPA	0.012	8.07×10^{-4}	−0.57
	0.03	8.82×10^{-4}	
Fenac	0.02	1.69×10^{-4}	−1.10
	0.05	1.98×10^{-4}	
MCPB	0.02	1.4×10^{-5}	+1.60
	0.05	1.5×10^{-5}	

TABLE 2.—*A comparison of* pK *values reported for the herbicides* [1]
[From: reference 1]

Reference	Method	pK							
		2,4-D	2,4,5-T	3-CPA	4-CPA	MCPA	MCPB	Fenac	Dalapon
1	Potentiometric	2.73	[illegible]	[2] 2.92	[2] 2.95	[2] 3.05	[2] 4.80	[2] 3.70	[2] 1.84
5	do	2.81	2.67						
6	do	2.95							
7, 8	Conductrometric			2.99	3.02				
9	do	2.64				3.05			
10	do	2.90	2.63		3.01	3.11			
11		2.93							
12	E.M.F			3.05	3.10				
13		2.78							
14	Literature review	2.81	2.64		2.96			3.57	1.58
15	do	2.69	3.14		3.16	2.99	4.86(60°)		
		2.80	3.46(60°)			3.45	6.21		
		3.22(60°)							
		2.73							

[1] Temperature=25° C unless otherwise stated.
[2] I=0.02.
[3] I=0.05.

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CHEMODYNAMIC PARAMETERS—ADSORPTION

Adsorption of pesticides to soil colloid surfaces has a major effect on the transport process and bioavailability. Accordingly, its importance has prompted many reviews (1–6), which should be consulted for a more detailed inquiry.

Adsorption reduces the concentration of a pesticide in solution. The resulting relative distribution between the adsorbed and solution phases depends on factors such as: the chemical parameters of the pesticide molecule (including pK_a or pK_b, size, shape, configuration, water solubility, and polarity); the soil properties (including the amount and type of clay and organic matter, surface area, soil structure, and soil pH); and soil temperature, water content, and salt concentration. Soil organic matter content is probably the most important single soil variant for nonionic pesticides.

Adsorption Model. The pesticide distribution represents a dynamic equilibration between adsorbed and solution (or desorbed) phases. The kinetics of these opposing reactions become important in modeling a leaching situation. Desorption may exhibit hysteresis, that is, a slower rate of release than that of adsorption, and some apparent irreversibility.

Adsorption equilibria have generally been described by the empirical Freundlich equation:

$$C_s = K\,C_w^{1/n} \qquad (1)$$

or

$$\ln C_s = \ln K + \frac{1}{n}\ln C_w \qquad (2)$$

or

$$\log x/m = \log K + \frac{1}{n}\log C_e \qquad (3)$$

where C_s is the amount (x) of adsorbed pesticide per unit amount of adsorbent (m); $C_w = C_e$ is the equilibrium solution concentration of the pesticide; and K and n are constants. The Freundlich adsorption coefficient, K, can be obtained by plotting log x/m versus log C_e, yielding a linear curve of slope $1/n$. The units of x/m and C_e are often in $\mu g/g$ and $\mu g/ml$, respectively. When C_e is 1 ppm (1 $\mu g/ml$), the corresponding value of log x/m is equal to log K. Deviation of the value of n from 1, a common observation, reflects the nonlinearity of the adsorption process. If n were 1, the K would be identical with the partition coefficient.

Adsorption Techniques. The typical method of conducting soil-pesticide adsorption studies is by the "batch" or "slurry" technique, such as that described by Weber (7). Air-dry soil is shaken with an aqueous pesticide solution until equilibrium is attained in the final solution concentration. This is measured by analyzing the supernatant after centrifugation. If degradation of the pesticide does not occur during this time, direct measurement of total radioactivity is usually the most convenient and accurate analytical procedure. To obtain the adsorption isotherm, a series of pesticide solutions are equilibrated with soil at constant temperature, and the amount of pesticide adsorbed is plotted as a function of equilibrium pesticide concentration.

Other methods used for adsorption studies have included continuous-flow equilibrium techniques (*8–10*) and a dialysis technique (*11*) in which the soil suspension was kept within dialysis tubing to retain lower molecular weight fractions (which would not be sedimented by centrifugation).

The adsorption coefficient, K, is commonly reported on a whole soil basis. Hamaker and Thompson (*3*) have compiled such data from many investigations; the results for individual pesticides have great variation, reflecting in part the various soils and experimental conditions. Distribution constants ranged from 0, for dicamba, to 14,000, for DDT. One method for standardizing adsorption data is based on the high correlation of adsorption with soil organic matter content. Thus the Freundlich K, divided by the percent soil organic matter content, yields Q, a value expressing the μg of pesticide adsorbed per gram of organic matter. Some authors (*3*) prefer the expression K_{oc}, i.e., adsorption based on organic carbon content, since this quantity is determined without the additional conversion factor (commonly 1.724) needed to derive soil organic matter content.

Relationships Between Adsorption and Pesticide Behavior. Osgerby (*12*) has attempted to model the availability of herbicides for plant root uptake. Of more direct environmental concern is the relationship between adsorption and leaching. This is discussed in part in the protocol describing laboratory leaching tests. Guth (*13*) plotted the penetration depth of several triazine and phenylurea herbicides against their K value and obtained a good correlation between these two parameters. He concluded that on addition of 20 cm "rainfall" to soil columns, pesticides whose K was greater than 20 would remain in the upper 2-cm zone, whereas those whose K was less than 1 would leach deeper than 30 cm. Decreasing soil pH tends to increase adsorption (and decrease leaching) for ionizable and polarizable compounds.

Adsorption coefficients, in addition to having merit in the prediction of pesticide mobility, may also be useful in estimating volatilization losses. This is discussed more fully in the protocol on volatilization.

PROTOCOL FOR ADSORPTION TESTS

Soils. Reference soils should be used if available. The same soils used in laboratory leaching tests are logical choices for adsorption measurements. Use a minimum of 4 soils, encompassing a range of soil organic matter contents and textural characteristics. Soil *pH* should be in the range of 4.5 to 7.5. If the pesticide structure and physical properties suggest its adsorption will be pH-dependent, testing should include a fairly acidic soil as well as a soil of neutral-to-slightly alkaline *pH*. If anticipated use warrants, a muck should also be tested.

In the absence of reference soils, those from regions of major pesticide use, including those soils available for field plot testing, would be logical sources.

Apparatus. Apparatus includes a high-speed centrifuge, shaker (preferably in a water bath unless ambient temperature is relatively constant and near 25°C), and appropriate sample containers. For nonanionic pesticides, glass containers are preferred. Because anions may adsorb to glass in signifi-

cant amounts, polypropylene containers are preferable for these. Other details of container design are not critical, except that the liners of screw caps should be Teflon. Test tubes with screw caps are convenient since they may be centrifuged directly, using an appropriate rubber adapter, and eliminate a transfer step.

Method. This protocol is based on the batch method. Additional methods and related details may be found in the references cited.

1. Soil preparation. Soils are sieved at less than 500 μm to remove stones and large plant fragments and coarse sand (500–2,000 μm). Although unlikely to directly contribute to adsorption, this quantity can be weighed and later accounted for when adsorption is determined on a whole soil basis. Soil characteristics (as discussed in the laboratory leaching protocol) should be reported here or elsewhere in the registration application. The water content of each air-dry soil should be measured by drying subsamples, at 110°C, to constant weight.

2. Pesticide solutions. Use at least 3 concentrations, 0.1, 1, and 10 ppm (if solubility permits), in aqueous 0.01 *N* $CaSO_4$ solution. The pesticide should be unformulated. In addition, a blank solution (0.01 *N* $CaSO_4$ only) should be included for each soil. Inclusion of a reference chemical whose adsorption coefficient is similar to the pesticide being tested is advisable.

3. Procedure. Use a ratio of 4 solution:1 soil, with the soil weight *based on* oven-dry weight (but do not use previously oven-dried soil). For example, 10 ml solution added to 2.5 g soil may be a convenient amount. Immediately after addition of the solution, agitate the container vigorously by using, for example, a vortex mixer. Subsequently, shaking is continued at a sufficient rate to keep the soil in suspension. The adsorption experiment should be run at 25°C, so a shaker water bath may be desirable. Each run should be duplicated, with additional replicates as needed for reliability of the data. Equilibration should be for 24 hr unless the chemical degrades rapidly. In such cases, only 2–4 hr may be needed, with possible analysis of both soil and equilibrium solution to verify the amounts of parent and breakdown products. After the period of equilibration, suspensions are centrifuged at high speed and an aliquot(*s*) carefully removed for analysis. Direct analysis of carbon 14 pesticides, by liquid scintillation techniques, is very convenient, but other appropriate methods may be used.

4. Data presentation. Average values of the adsorbed pesticide, x/m, should be plotted as a function of equilibrium concentration, C_e, according to Equation 3. C_e is obtained directly in $\mu g/ml$ (or ppm) from the solution analysis. The amount of pesticide adsorbed per gram of soil, x, is obtained by:

$$x = \frac{(C_o - C_e)\,V}{m} \qquad (4)$$

where C_o is the starting concentration ($\mu g/ml$), V is the solution volume (ml), and m is the soil weight (g). The Freundlich constant, K, and the constant n can be evaluated from the adsorption isotherm. At $C_e = 1\ \mu g/ml$, the corresponding value of x/m should be reported as K. The adsorption coefficient may *additionally* be reported as Q or K_{oc} by the equations:

$$Q = \frac{K \times 100}{\%\ \text{soil organic matter}} \qquad (5)$$

or

$$K_{oc} = \frac{K \times 100}{\%\ \text{soil organic carbon}} \qquad (6)$$

The final tabulation of data should therefore, include K, $1/n$, and Q or K_{oc} (if determined) for the pesticide in each soil. Reference chemical data is included as well.

5. Desorptive studies. Experiments should also be carried out to study the desorption of pesticides. Studies could be accomplished by adding water to soil containing absorbed pesticides, equilibrating, and then analyzing the concentration of pesticide in solution. The desorption data may also be treated by the Freundlich isotherm.

6. Optional experiments. Basic studies on adsorption could be expanded, but are not required, to include other temperatures, additional soils (especially where soil *pH* varies or is modified), time to reach equilibrium, and additional reference compounds. A broader concentration range may be used in better defining the adsorption isotherm. Giles (*14*) offers useful information on interpretation of adsorption mechanisms based on the shape of the isotherm.

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HYDROLYSIS STUDIES

Organic pesticides may enter natural waters via such routes as direct applications to control aquatic weeds, trash fish and aquatic insects; leaching and runoff from agricultural lands; drift from aerial and ground applications; discharge of waste waters from manufacturing plants; and discharge of waste waters from cleanup of equipment used for pesticide formulations and application. Thus, a knowledge about the mobility and degradation of pesticides in aquatic environments is necessary because it has a direct bearing on their persistence in waters and soils. Pesticide hydrolysis data will be helpful in understanding pesticide degradation in water; the impact on the choice of formulation technology, transportation and storage procedures; and pesticide disposal (*1–3*).

The following is a suggested protocol for the study of chemical hydrolysis of pesticides in aqueous environments.

Materials and Methods. A radiolabeled preparation is a preferred way of studying the hydrolysis of pesticides. The position of the radiolabel must allow detection of the hydrolysis products. In such cases, the identification of hydrolysis products, disappearance of the parent pesticide and appearance or disappearance rates of the hydrolysis products can be readily determined through thin-layer chromatography (TLC), TLC-radioautography, liquid scintillation counting (LSC) and related radiotracer methodologies.

Hydrolysis studies can also be conducted using nonradioactive compounds. If a nonradioactive pesticide is used, the rate and extent of its disappearance in an aqueous system may be determined by appropriate analytical procedures, such as gas liquid chromatography (GLC), spectrophotometric determinations, etc.

Hydrolysis experiments will be conducted at constant temperature in darkness. An initial range-finder probe may be run to choose appropriate time intervals for the final experiment. At least 4 sampling times, with at least one observation made after half of the parent pesticide is hydrolyzed, are recommended.

Temperatures.—Three temperatures should be used: 25°C (77°F), 35°C (95°F) and 45°C (113°F). A certain degree of extrapolation of the rates obtained at 25°, 35° and 45°C is possible. If very little or no hydrolysis occurs at 45°C, experiments may be carried out at higher temperatures.

Concentration of the Parent Pesticide. Two concentrations are recommended. The higher concentration to be used depends upon the limits of pesticide water-solubility and the lower concentration used based on the sensitivity of the analytical method to detect the parent pesticide and/or its hydrolysis product(s). In any event, the higher concentration should not exceed 250 ppm. The lower concentration should be approximately 1/10 that of the higher concentration. The inclusion of 2 concentrations in this protocol will lend support to the assumption that, under the present experimental parameters, first-order kinetics describe the hydrolysis reaction.

pH Values. Three pH values will be studied: 3, 6 and 9. The pH measurements on 49 different soils demonstrated that this range would include a majority of agriculturally important soils. The pH and ionic strength of the reaction solution should be maintained by the use of suitable buffers. (*4*).

Experimental Procedures. All solutions should be incubated at the desired temperatures in sterile sealed glass containers for appropriate times in darkness. For analysis, each solution should be extracted with appropriate organic solvents, if required, and the qualitative and quantitative determinations on the parent pesticide and/or its hydrolysis products should be performed using appropriate analytical procedures. Each experimental point should be studied in duplicate.

Calculations and Discussion. The hydrolysis data should be treated with simple first-order kinetics, and the rate constant and half-life should be calculated (*5*). The activation energy of the process should be determined from the temperature dependence of the rate constant. In cases where the first-order rate law is not applicable, the hydrolysis data should be treated with the second-order or other kinetic scheme. A study by Gomma et al. is one example of the presentation of hydrolysis data.

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PHOTOCHEMICAL STUDIES

The action of sunlight may destroy or chemically alter pesticides in the environment. The rate of breakdown and the potential of the products to contaminate the environment influence the usefulness of the parent compound. Environmental photochemistry is complicated by the possible interaction of the reacting molecule with many environmental components. Laboratory experiments are necessary to obtain comparative data under standardized conditions and to understand the effect of factors that might modify the rates and products of reaction.

General Procedures. The enumeration below lists the stages of a model photochemical investigation. States 1 through 6 indicate general procedures suitable for an investigation of solution phase photolysis.

1. Measurement of the absorption spectrum of the unknown compound to identify the spectral regions that might cause photochemical change.

2. Selection of light source, wavelength range and apparatus for irradiation.

3. Choice of solvent and concentration of the reactant.

4. Irradiation of the mixture, with periodic sampling, to measure rate of disappearance of the starting material and observe the appearance of products.

5. Quantitative and qualitative analysis of products after a selected period. Identification of unknown compounds.

6. Study of modifying factors; for example, effect of physical state, effect of triplet and other sensitizers, effect of oxygen, effect of pH and solvent, effect of adsorption on soil or silica.

Applicable Theory. There are 2 fundamental laws of photochemistry: The first is the Grotthus-Draper law which states that only the light absorbed by a molecule is responsible for reaction. The second is the Stark-Einstein law which states that the absorption of light by a molecule is a one-quantum process, so that the sum of the primary quantum yields, must be unity (*1*).

Elaborate experimental techniques are necessary for the investigation of quantum yields. The area that has received major attention from the theoretical standpoint is that of atmospheric photochemistry, where consideration can be given to the reactions of low concentrations of organic molecules in the vapor phase (*2*). Quantum yield measurements of organomercurial compounds in water lead to predictions of reaction rate in reasonable agreement with those determined environmentally in natural waters (*3*).

In the environment, "sensitized" processes may be important. In such a case, light is absorbed by a "sensitizer" molecule which adopts an excited electronic configuration. This excited electronic state possesses a relatively long life time during which the molecule can transfer energy to a molecule of another species which does not absorb light in the same region as the sensitizer molecule. Thus, a molecule which does not absorb light in the visible region of the spectrum may undergo reaction in the presence of a sensitizer absorbing at longer wavelengths (*4–7*).

Rates of photochemical reaction are not usually influenced by temperature to any appreciable extent; however, the study of rate of photolysis is complicated by the formation of photoproducts. These absorb light in competition with starting material. It is therefore more meaningful to measure rates of disappearance until not more than 20 percent of the starting material has been consumed and to use low concentrations. At high concentrations polymeric materials may be formed. Concentrations to be expected in the environment will be low (10^{-2} to $10^{-6}\mu$); therefore, studies at low concentrations will provide more relevant information. Disappearance rates of organic compounds as a result of photolysis in very dilute aqueous solution can be represented by a first-order rate law.

Influence of Environmental Factors. Water is the predominant solvent for the study of photochemical reactions in the environment. Organic solvents frequently used for photochemical studies include methanol, acetone, benzene, isopropanol, hexane, acetic acid, ethyl acetate, and cyclohexane. The ability of some of these solvents to transmit light of shorter wavelengths is limited. Methanol or hexane, however, are examples of solvents which transmit an appreciable amount of energy at 220 nm.

Benzene and acetone absorb light and are capable of acting as sensitizers. The presence of acetone in aqueous solutions may be sufficient to cause photosensitized reaction of other molecular species (*8, 9*). The presence of sensitizers in "natural" waters has long been suspected and there is increasing evidence that, although some photochemical reactions do not occur in distilled water, photoproducts may be obtained in "natural" water as a consequence of sensitized reaction (*10*).

Solvents do not merely serve to dilute the reacting species. Many can participate in the photochemical reaction. Irradiation of *o*-chlorobenzoic acid proceeds by the formation of a radical or excited species which reacts with the solvent. In methanol, this radical abstracts a hydrogen atom and benzoic acid is the major product. Methanol, isopropanol, hexane, and a number of related solvents function as hydrogen donors. By contrast, benzene forms a new carbon-carbon bond and photolysis of *o*-chlorobenzoic acid in benzene gives *o*-phenylbenzoic acid (*11*).

In addition to the influence of solvent, the presence or absence of oxygen also influences the course and products of reaction. In practice, the total exclusion of oxygen requires rigorous procedures that are rarely adopted (*1*), but, by bubbling oxygen-free nitrogen through the solution, the effect of anaerobic conditions can be studied. Oxygen adds to radical sites and competes with other reagents for these sites. Comparison of the photolysis of DDT and DDE under oxygen and nitrogen shows that photo-oxidation reactions predominate in the former case (*12*). Oxygen is itself a diradical and, in light, it is capable of reacting with other systems, energy transfer to oxidizable subtrates, direct free-radical reactions or excitation to an activated species (*13*).

Photochemical studies of pesticides have been performed on thin layers of pure material deposited on glass plates, on silica-gel coated glass plates or on thin layers of soil. There are few reported studies of photolysis on soil, but the rate of photolysis appears to be slower than that on silica under comparable conditions (*14*).

Photolysis has been done on silica-gel coated plates and the products have been subsequently examined by thin-layer chromatography. However, changes in absorption spectra have occurred in pesticides deposited on silica-gel (*15*). Such spectral changes are a general phenomenon and depend on the nature of the compound (*16*). Silica is an acidic polar medium. It interacts with organic compounds through hydrogen bonding and dipole-dipole forces. Soils and clays might be expected to exhibit weaker interactions, if any, and there is little information on the spectral changes to be anticipated when pesticides are deposited on soil, clay or other surfaces. Therefore, the extension of photochemical information obtained by studies on silica-gel coated plates to environmental situations should be carefully interpreted.

Experimental Techniques. Although the argument may be raised as to the value of "model" or laboratory photochemical experiments in providing a guide to environmental behavior, valuable information may be obtained using a relatively limited range of conditions. Environmental samples are exposed to light under a wide variety of conditions: absorbed on dust; as solids on foliage or soil; dissolved in water; dissolved in organic films on water surfaces; in the vapor phase, etc. Studies of photochemical behavior under such a variety of conditions would be impractical. Discussion of environmental influences requires basic information that can only be obtained in controlled experiments.

Photolysis of pesticides has been investigated in vapor, solution and solid phases. Descriptions of experiments on vapor-phase photolysis of pesticides are limited in number (*17, 18*). Discussion has therefore been limited to photolysis of solids and solutions.

Natural sunlight and artificial light sources have been used for the study of photochemical reactions. The value of experiments in sunlight is that the reactions observed are those that may be expected to occur in the environment. Unfortunately sunlight is extremely variable. Wavelengths and intensities depend on latitude, climate, horizon geometry, near ground distribution of aerosol and gaseous pollutants and many other factors. Thus it is difficult to ensure the reproducibility of experiments performed in natural sunlight, and artificial sources are necessary to standardize conditions and measure rates of reaction. If studies are performed in natural sunlight, it is usual to carry out parallel experiments with artificial light sources for purposes of comparison, control and precise recording of experimental variables. Arc lamps and fluorescent lamps are preferred sources for photochemical experiments since they are good sources of ultraviolet radiation.

The mercury arc is one of the most frequently used artificial light source for photochemical experiments. There are 3 types of mercury arc: low-pressure, medium-pressure

and high-pressure. The light from the low-pressure or "resonance" lamps is rich in 253.7 nm radiation (also in 184.9 nm radiation if a "suprasil" quartz envelope is used). They require no special cooling and are often used for photochemical research. The medium-pressure mercury arc is a more convenient source of wavelengths above 260 nm.

Protocol for an Environmental Study. Rate studies should be conducted initially at a concentration in the range of 5 ppm (or lower) in distilled water at 25° C. The use of radioactive material may facilitate the investigation of photoproducts. Periodic sampling should be carried out from zero time in order to measure the rate of disappearance of the parent compound and decay of major photoproducts. Conditions should be essentially aerobic, i.e., excess oxygen should be present during the irradiation. (It may be necessary to bubble air through the liquid.) An estimate of the material balance at the completion of the experiment will indicate whether loss of volatile material has occured. It may be necessary to trap volatile products for identification, if these represent a significant proportion of the photoproducts.

Borosilicate vessels are adequate for these studies. A reactor, such as the Rayonet photochemical reactor, permits the exposure of several tubes under uniform conditions of illumination. Alternatively, reaction vessels utilizing light sources housed in a cooled well may be used (*19*). The ability of glassware to transmit light varies from batch to batch. For calibration of the performance of light sources and glassware a chemical actinometer is recommended.

The photolysis should be continued until sufficient data points are obtained to indicate the half-life of the parent compound. In each experimental design a reference substance should be included to obtain a relative indication of half-lives under similar conditions of illumination.

Studies should be carried out in the laboratory under controlled conditions using an artificial light source emitting wavelengths greater than 280 nm, a source equivalent to natural sunlight, or in natural sunlight. Efforts should be made to identify or characterize products that do not appear to be of transitory nature.

Since differences in products and reaction rates may be observed between sensitized and unsensitized photochemical reactions (*20*), experiments should also be performed using distilled water containing 2 percent acetone. Acetone is a triplet sensitizer and may mimic the sensitizing effect of (unknown) dissolved substances present in natural waters which are also capable of sensitizing the reaction. Studies a high and low pH in buffered aqueous solution may also be required to provide further information on stability to light.

Since the quantities of material obtained in the rate study may be inadequate, product identification may be accomplished in parallel experiments. Changes in experimental conditions, such as wavelength of light and concentration of starting material, will influence the course of the photochemical reaction. However, such changes may afford improved yields of photoproducts for structural studies. The objective of parallel experiments is primarily the identification of photochemical products observed in the course of the rate study.

Additional photochemical studies on silica-gel or soil coated thin-layer glass plates are optional and may be carried out for a particular compound, if desirable. Caution should be exercised in interpreting these data.

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LABORATORY LEACHING STUDIES

Leaching of pesticides implies the movement of these chemicals through soil, usually by a mass transport process. The depth and concentration of a pesticide within the upper 20 cm has particular implication for pesticide efficacy. Of greater environmental concern, however, is the possibility of movement to and contamination of groundwater or drainage effluent.

Degradation, which occurs simultaneously with movement, will attenuate leaching, although this is rarely studied as a simultaneous process (*1–4*). Other important factors affecting pesticide movement are adsorption, net water infiltration, flow rate, diffusion and dispersion, and pesticide solubility (*5–7*). Adsorption is perhaps the dominant factor. Increased pesticide adsorption has been correlated with reduced leaching (*6*).

No laboratory study can, in itself, absolutely predict leaching; however, laboratory leaching tests should provide a quantitative or semiquantitative index of the *inherent mobility* of pesticides within the soil.

Basic Laboratory Techniques. The two basic techniques for direct leaching measurement are soil TLC and soil columns.

Soil TLC. Soil thin-layer chromatography As with soil columns, it has a fundamental analogy in conventional chromatography. Unlike columns, however, extensive basic research on water movement relationships has not been conducted in soil TLC systems. Helling (*9*) has shown, however, that water moves up a soil TLC plate by unsaturated flow and that the advancing waterfront decreases in speed as a linear function of the distance squared. Both characteristics are typical of "normal" soil relationships.

Among the advantages of soil TLC are: (1) Relative simplicity, in terms of equipment and techniques; (2) good reproducibility of soil layer characteristics and pesticide mobility results; (3) direct analysis via autoradiograph, radiochromatographic scanning, or bioassay for a continuous profile of pesticide movement (when leaching has concluded); (4) ease of distinguishing both mass transfer and diffusion components; (5) small requirements in amounts of chemical and soil, and in lab space; (6) use of multiple applications of pesticides on a single TLC plate; and (7) a convenient index of quantifying pesticide leaching, the R_f value, or movement of the chemical relative to the distance infiltrated by water.

The disadvantages of soil TLC include: (1) Inability to easily control water flux; (2) less satisfactory adaption to volatile materials such as fumigants [although Nemagon ® and other diffusive compounds have been studied in a soil TLC system (*10*)]; (3) a more homogeneous soil matrix than occurs in nature [although disturbed soil columns, as used in virtually all laboratory tests for leaching also differ from nature, as shown by movement of inorganic solutes (*11*)]; and (4) less satisfactory adaption to long-term leaching/incubation experiments (largely because pesticide diffusion often becomes quite significant in the relatively small leached zone).

Movement in soil TLC is usually measured as the frontal R_f of a spot or streak, with the R_f value shown to be independent of sample size (*9*). For comparison of mobility, a scheme of 5 mobility classes based on R_f in a standard soil, Hagerstown silty clay loam, was proposed (*8*), as shown in Table 1.

TABLE 1.—*Pesticide mobility classification for soil TLC plates*

[From: reference 8]

	Class				
	1	2	3	4	5
R_f range	0 to 0.09	0.10 to 0.34	0.35 to 0.64	0.65 to 0.89	0.90 to 1.
Description	Immobile	Low	Intermediate	Mobile	Very mobile

This mobility classification does suffer the disadvantage of being linked to one specific soil; however, classifying a pesticide in this scheme *based on the soil being tested* should qualitatively indicate leaching behavior in that soil.

There is a trend toward the modeling of pesticide transport in the environment. The input related to leaching may be presented in part either by adsorption data, such as Freundlich constants, or by laboratory leaching data. If relationships between soil TLC R_f values and classical adsorption constants become sufficiently well defined (*8, 10, 12–15*), either source may be useful in the predictions. It appears that soil TLC data should reflect more factors affecting leaching than adsorption, and therefore may be a better model input. Presently, soil TLC data are simpler to acquire than complete adsorption data.

Soil Columns—Some of the specific advantages of soil column experiments include: (1) Previous, widespread use (a questionable advantage, given the variation in technique); (2) flexibility in design (again, not always advantageous from the standpoint of standardizing results); (3) ease of studying soil water relationships; (4) wide choice of application method, amount and formulation of applied pesticide, and method of analysis; (5) adaptability for use with volatile pesticides; (6) the possibility of using intact soil cores (very rarely done); and (7) the ability to maintain an experiment over a long time-span, if desirable.

Disadvantages of columns include: (1) Difficulty of standardizing column packing and other techniques; (2) some awkwardness in recovering the column soil for subsequent analysis after leaching (the use of segmented columns does facilitate this, however); (3) inherent discontinuous analysis of both effluent and column; (4) the potentially large amounts of soil and chemical required; (5) the large laboratory space required for extensive testing; (6) the inability (normally) of replicating or running several compounds on one column during one run; and (7) lack of an accepted "yardstick" for quantitatively reporting leaching data.

Protocol for Soil TLC Study. The recommended protocol is based on the use of soil TLC, i.e., leaching of pesticides applied to thin soil layers by an ascending chromatographic technique. A brief supplemental discussion of soil column methods follows the soil TLC protocol.

Soils. Use a minimum of 4, encompassing a range of soil organic matter contents and textural characteristics, for example, sand (agricultural), sandy loam, silt loam, and clay or clay loam. Organic matter content in at least one of the light textured soils should not exceed 1 percent. Soil *pH* may range from 4.5 to 7.5. If the pesticide is acidic or if application to alkaline soils is anticipated, at least one soil should be greater than or equal to *pH* 7.0. If anticipated pesticide use warrants, a muck should also be tested.

If reference soils become available, these should be used. In their absence, soils from regions of major pesticide use are reasonable choices. Soils available for field plot testing would be logical sources.

Apparatus. Standard thin-layer chromatography apparatus is preferable, including 20 x 20 cm glass plates, spreader (preferably variable thickness), chromatographic developing tank, etc. A satisfactory variation is the Gelman® chromatography apparatus in which soil plates are developed horizontally using a filter-paper wick. Since pesticide location is conveniently determined by use of radioisotopes, either no-screen medical X-ray film or a TLC radioactivity scanner is advisable.

Method. Additional descriptions of the soil TLC method appear in the references (*8, 9, 10, 14*).

1. *Soil preparation.* Soils are prepared by sieving to remove stones, coarser sand fractions, and large plant fragments. If crushing or grinding is involved, this step should reduce soil aggregate size but not create more fine particles (silt and clay) than originally present. Soils that are classified as sand, loamy sand, sandy loam, sandy clay loam, or sandy clay should be sieved at less than 500 μm. In general, this includes soil containing more than 50 percent sand. Finer textured soils (loam, silt loam, silty clay, etc.) should be sieved at less than 250 μm. This removes the coarse (500–2,000 μm) and medium (250–500 μm) sand fractions. Earlier testing has shown that removal of a portion of the sand did not affect pesticide mobility but did aid in achieving a more uniform soil layer. In reporting data, specify the size sieve used. Also, soil characteristics (soil clay and organic carbon contents, soil *pH*, field moisture capacity, and perhaps other properties) should be reported, specifying whether the analyses were made on whole soil or that sieved at, for example, 250 μm.

2. *Plate preparation.* Water is added to a sieved soil until a smooth, moderately fluid slurry is attained. Application of the soil slurry to clean glass plates should be done quickly enough to prevent particle size segregation. This is more likely to occur in coarse textured soils than in finer ("heavier") soils. In soils where the proportion of coarser sand is high, soil TLC plates may be more satisfactorily prepared by smoothing a direct application of soil with a glass rod. Layer thickness can be controlled, in this case, by affixing multiple layers of masking tape along the plate edges. Soil layer thickness should be 500–750 μm for soils sieved at less than 250 μm and 750–1000 μm for soils sieved at less than 500 μm. If a bioassay method for spot detection is employed, layer thickness should be 1000 μm to resist soil drying. Plates are air-dried before use.

3. *Pesticide application.* At least 4 soil TLC plates should be used per soil for replicating results. Soil plates in which the layer thickness varies within the zone of leaching should be discarded because water movement may be irregular and pesticide leaching patterns distorted. Application of "reference" chemical standards in addition to the pesticide(*s*) being tested is strongly advised to afford relative comparisons of mobility with other pesticides whose laboratory and field behavior (usually on other soils) is already known. Reference chemicals should include at least one mobile ($R_f \geq 0.65$ in a medium textured soil) and one less mobile (R_f 0.10–0.64) compound. Extensive lists of relative pesticide mobilities have been published (*10, 16*); relatively mobile compounds are in classes 4 and 5, less mobile compounds are in 2 and 3. In addition to the parent pesticide and reference compounds, significant degradation products should also be tested for leaching. Since 20 x 20 cm soil TLC plates can accomodate up to 7 or 8 individual applications, when visualization is by autoradiography, it is normally convenient to run all compounds (pesticide, metabolities, and reference chemicals) on each plate. Application rates on soil TLC are not directly comparable to field rates, but should be kept as low as practical, for example, 1 μg. "Aged" soil, i.e., pesticide-treated soil incubated previously as part of the aerobic metabolism test, can be extracted; extracts can then be tested directly for the presence of mobile components.

4. *Plate development.* A horizontal line is scribed through the soil layer 11.5 cm above the base. By spotting pesticides at 1.5 cm, the compounds are then leached 10 cm. Plates are immersed with the base in approximately 0.5 cm water in a closed chromatographic chamber and removed when water has risen to the scribed line. When compounds known to photodegrade are used, the leaching should be conducted in darkness.

5. *Pesticide visualization.* Soil TLC plates are then air-dried, unless volatile compounds are present. In this case, plates can be frozen immediately after leaching to reduce loss of the pesticide. If radioactive compounds are used, spot movement is determined from radioautograms made by direct exposure to no-screen medical x-ray film exposed for at least three days. Alternative methods, such as chromatographic scanning or scraping segments from the chromatogram and determining radioactivity through scintillation counting, may be necessary. Bioassay or chromogenic reagent methods of pesticide visualization on soil TLC are available (*17–20*), but are less satisfactory.

6. *Data presentation.* For each soil, mobility data is reported as the average frontal R_f ($\pm$ standard deviation) of a spot or streak; i.e., if a compound moves 5.5 cm, its R_f is 0.55. The R_f of relatively immobile compounds should be corrected for the initial spot radius. Center R_f (the usual measurement for conventional TLC) may additionally be reported for compounds leaching as spots, but must be clearly distinguished from frontal R_f.

7. *Data documentation.* In addition to R_f data, a representative photograph or diagram of the leaching patterns for each soil should be submitted.

Protocol for Soil Column Study. Examples of many variations in soil column technique appear in an earlier review (*6*). Since it is not feasible to reproduce the complexities of the field in the laboratory, the techniques used in column leaching experiments should be sufficiently simple and standardized to afford reproducible relative mobility data for pesticides in a variety of soils. As with soil TLC, soil column-derived values must eventually be calibrated against the "real world" experience of field data.

Soils. Use the same selection criteria described for the soil TLC protocol.

Apparatus. Since analysis of column experiments should involve the soil itself, as well as percolate fractions, the use of a segmented column (glass or metal) is convenient. Plastic materials should be avoided unless prior testing has shown that the pesticide (and its metabolites) are not significantly adsorbed on the column walls and that plasticizers eluting from the column do not interfere with analyses.

In one protocol (*21*), segmented columns are filled to a height of 30 cm with air-dried soil. Each segment is 2 cm long x 4 cm inner diameter, permitting rather discrete analysis of the pesticide distribution. Besides 15 shorter pieces, the column also has one 10-cm section. The soil surface is usually covered after pesticide addition, with a disc of glass-fiber filter paper, sintered glass, or glass wool to minimize disturbance of the soil and uniformly distribute water applied during leaching.

Another modification of this apparatus, Harris' (*22, 23*) use of stacked aluminum rings, 2.5 cm x 7.6 cm (I.D.), was used to evaluate comparative mobilities of 28 herbicides (*22*) and 11 insecticides (*23*). The soil column length was 18 cm. The use of longitudinally slotted (*24*) or split columns have been convenient for bioassaying columns *in situ* after leaching.

Additional descriptions of soil column leaching apparatus appear elsewhere (*25*). A major precaution in the preparation of columns is the necessity of uniform packing. A known weight of 2-mm-sieved, air-dried

soil is added slowly, for example, with column vibration. Bulk density should be measured and reported and should be nearly the same for all columns of the same soil.

Method. The parent pesticide, major metabolite(s), or previously aged (via the aerobic metabolism study) radioactive pesticide is added uniformly via treated soil, or in a minimum amount of solvent, to the top of the air-dry soil column. The amount of parent added should correspond to the highest recommended rate for a single application. Although a greater effluent volume will obviously be collected if the soil column is initially at field capacity, tests have shown (*26*) that penetration depth of several herbicides was the same in dry as in moist soil columns. With the appropriate filter pad in place, water or 0.01 *N* $CaSO_4$ is added at a rate which does not exceed the infiltration capacity of the soil.

Sufficient 0.01 *N* $CaSO_4$ solution should be added to achieve at least one column volume of effluent. (Specify the amount used and the period over which leaching occurs.) It is anticipated that by leaching a 30-cm soil column with 20 cm of solution, 1 to 3 column volumes of water effluent will elute from the column. Since this amount depends on the moisture retention characteristics of the packed column, the ⅓-bar moisture content and the volume of effluent collected (after drainage has stopped) should be reported.

Reports of leaching tests should be accompanied by data on at least one reference chemical with mobility characteristics similar to the parent pesticide under examination. Evaluation of leaching results would be enhanced, however, if reference chemicals showing significantly greater and less mobility are also included.

Leaching data should include analysis of effluent fractions and the soil itself. In a 30-cm column, individual segments should be approximately 5 cm each. At least 3 replicate columns should be run per test. If tests on aged soil have demonstrated a mobile constituent, efforts should be made toward its chemical identification.

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FIELD LEACHING STUDIES

Reviews of pesticide dissipation from soils published in recent years have referred to the leaching of pesticides as measured in the field (*1–3*). Leaching of pesticides implies the displacement of the chemical in the soil by water. About the only measure of pesticide leaching that many field studies provide is the depth of penetration as measured by soil sampling. Usually the dissipation of pesticides from soils includes the processes of volatilization, degradation and all its ramifications, and photolysis as well as leaching. Consequently, depth of penetration studies provide very little information concerning the displacement of chemicals by leaching relative to other modes of dissipation. Nor do they provide measurements of water contents of the soil profiles, the flux of water, rainfall, applied water, etc., that are necessary to quantity the displacement process.

Intermittent sampling of soil profiles to assess the time and spatial displacement of a chemical is often used as a measure of leaching patterns. Usually such measurements assume that any leaching that has occurred in the interval will be manifested by adsorbed pesticide at the depth of interest. If the chemical is absent, the assumption is made that penetration to that depth has not occurred.

A logical question to be asked before adopting a specific protocol for leaching investigations is whether the leaching experiment should be conducted for those conditions under which the pesticide is normally used, or whether the investigation should be designed to examine whether the pesticide can be leached.

Adopting the former argument involves a definition of what "normal" conditions are and, therefore, leaves open the questions which are usually never characterized; i.e., what were the actual conditions and how are these interpreted in terms of the broad range of conditions that might occur. Further, other processes that may also account for the dissipation of the pesticide, such as water content of the profile, temperature, rainfall, etc., confound the relation between the amount of chemical applied and the depth of penetration.

In contrast, leaching investigations can be designed to examine the leaching characteristics of the chemical under conditions which are not usually considered normal, but reasonable. Such conditions could be more adequately characterized and the behavior of the chemical more adequately interpreted in terms of other processes that contribute to its dissipation (for example, any process which reduces the concentration that could exist in a mobile form).

A review of the literature suggests a protocol should be developed which adopts the latter viewpoint. Clearly, some procedure should be adopted which improves on procedures currently described and followed by most investigators. If such improvements are not made, the evaluation of pesticide leaching will continue in a haphazard manner and the application of theoretical analysis will not materialize.

Protocol for Field Leaching Studies. The protocol outlined below could be used for field leaching tests should such tests be deemed advisable. It is presented as a guide although modifications could be made where conditions suggest such modifications would be desirable.

I. *Location.* The test locations could be selected according to the requirement of the pesticide use.

II. *Time Period.* The tests should be initiated when the pesticide is normally applied. Since the purpose of the test is to examine leaching characteristics, a short period of intensive monitoring is suggested to examine the leaching characteristics immediately after application before other dissipative processes become dominant, i.e., a period of 1 to 2 wk. Less frequent monitoring for longer periods of time would yield information on field dissipation which might include other processes, such as degradation, that become more dominant with time.

III. *Experimental Design.* A. Three types of treatments are being suggested for comparative purposes. However, the difference between the 3 treatments is such that for short-term experiments only one—where excessive water and a pesticide application rate larger than the normal rate is used—may be meaningful. Two plots in each treatment are suggested to provide some assurance that should unusual conditions exist either in the conduct of the test or the soil, the test will not be lost.

1. Excessive irrigation—3x pesticide. Two plots which receive high pesticide application rates and excessive water for the spe-

cific purpose of testing the tendency of the pesticide to leach.

2. Normal irrigation—rainfall—1x pesticide. Two plots which receive recommended rates of pesticide use and water applications based on normal yearly rainfall amounts or where irrigation is practiced (and rainfall is low).

3. *Normal rainfall—3x pesticide.* Two plots which receive only normal rainfall (for areas where no irrigation is practiced). This treatment is more in the nature of a field dissipation study and is included only for comparative purposes.

B. The area selected should be level and free of vegetation. The soil surface should be lightly tilled before application of the pesticide. Following application of the pesticide, the chemical should be tilled into the upper 4 in. of soil and mixed as thoroughly as possible. A rototiller can be used for this.

C. The form in which the pesticide is applied would be determined by the formulation. Uniformity of application is important and can best be achieved either by spraying on a pesticide soution or by uniformly spreading a dry mixture. In some cases, the pesticide could be mixed in a large volume of water outside the plot and applied as the first irrigation to the plot. This procedure has been successfully accomplished by mixing the chemical with water in a plastic swimming pool set up outside the plot and pumping the mixture to the plot. The volume of water used was based on the volume of water that would cover the test plot to a depth of 2, 3, or 4 in.

D. Recommended application rates.

1. On the heavily irrigated plots the recommended rates would be 3x the recommended rate.

2. On the plots where irrigation is used to simulate normal rainfall or where irrigation is the sole main source of water, the recommended rate is 1x.

3. On normal rainfall plots the recommended pesticide rate is 3x.

E. The plot size is not crucial except that a larger area minimizes the effect of lateral flow in the region of instrumentation and provides a larger sampling area for repeated sampling. It also reduces the effect of unusual soil variability. An area 7 m sq (or 20 ft sq) has been found useful. The plot is surrounded by an artificial barrier, such as planking (1 in. x 12 in.) which is sunk into the soil surface 6 to 8 in., back filled on both sides, and tamped to prevent the escape of water from within the plot. This design is used so that water may be ponded on the surface. However, a good sprinkling system, capable of applying the water uniformly, could also be used in lieu of the barrier. In this case, a low soil berm may be used to contain any water which might run off. Plots should be separated by 3 or more meters.

IV. *Instrumentation.* All instruments in the 7 m sq plot should be located in an area 3 m x 3 m.

A. *Suction probes.* Soil solution is obtained during the leaching process by means of suction probes. Three probes at each depth are suggested to provide sufficient solution for analysis and to overcome some of the variable leaching characteristics of the profile. A minimum of 2 probes should be installed.

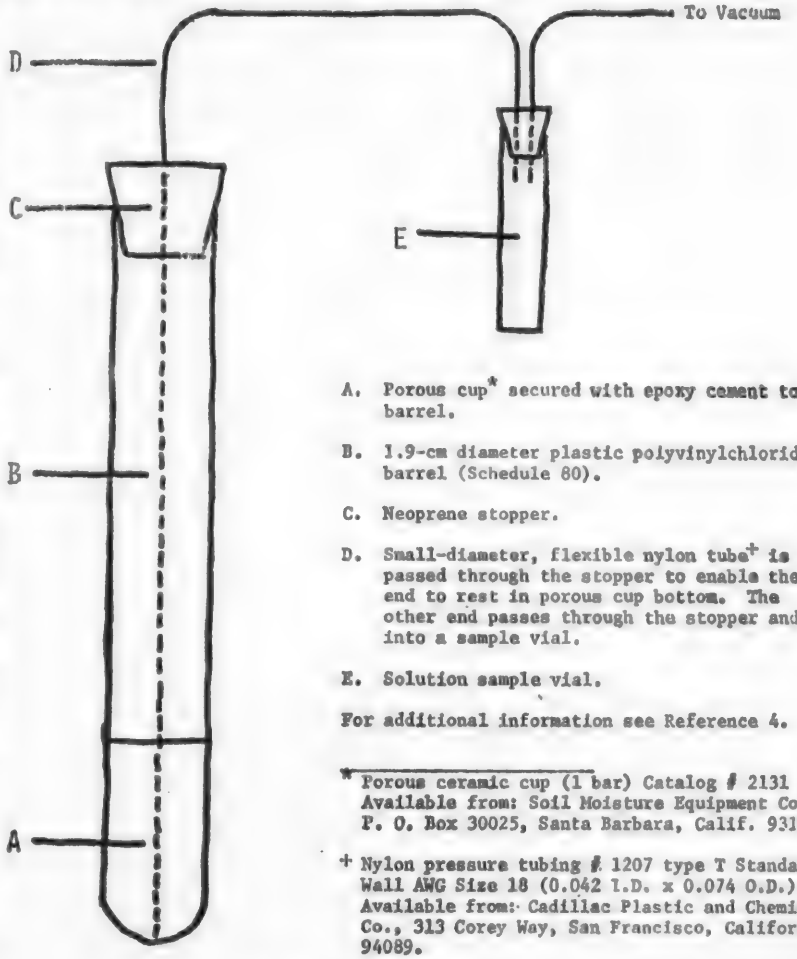
To examine the movement of the pesticide, should it occur, the probes should be installed at 15, 30, 60, 90, and 180 cm. In soils where shallow water tables are a problem, the spacing at the deeper depths could be eliminated or compressed. Good installation procedures suggest that an ordinary wood bit of the same diameter as the suction cup be used to make the hole for the suction probe. The bit is welded to a rod for ease of boring.

The suction probe can be constructed from PVC tubing (Schedule 40) to which the ceramic cup is glued. (See Figure 1.) Should the PVC tubing be deemed undesirable, copper tubing has been used. However, in general the soil solution does not come in contact with the PVC tubing which merely acts as a holder of the cup, and the PVC is easier to work with and less expensive. The ceramic cups are available from commercial suppliers and should be washed with a solution of dilute HCl several times and washed with distilled water before use.

The probes should be installed when the soil is moist and easy to work. Once the probe is in place, the soil should be tamped firmly around the barrel to a depth of 6 in. to prevent channeling.

After the probe has been installed a tube of small bore (⅛ in. to 1/16 in. in diameter) and of sufficient length to extend from the bottom of the ceramic cup to the edge of the plot, is inserted. The insertion takes place through a rubber stopper which seals the top of the probe. If nylon or other plastic tubing is undesirable, ⅛-in. O.D. copper tubing can be used.

At the edge of the plot the tubing enters a collection receptacle through the stopper. Passing through this same stopper is another tube of ¼-in. diameter which is connected to a vacuum source of 0.3 atm. Usually a manifold can be constructed to supply vacuum to all the probes in a plot. Glass vials of various sizes fitted with screw caps lined with foil have been used successfully. Liquid scintillation vials of 30–50 ml capacity are quite satisfactory.



A. Porous cup* secured with epoxy cement to barrel.

B. 1.9-cm diameter plastic polyvinylchloride barrel (Schedule 80).

C. Neoprene stopper.

D. Small-diameter, flexible nylon tube+ is passed through the stopper to enable the end to rest in porous cup bottom. The other end passes through the stopper and into a sample vial.

E. Solution sample vial.

For additional information see Reference 4.

* Porous ceramic cup (1 bar) Catalog # 2131 Available from: Soil Moisture Equipment Co., P. O. Box 30025, Santa Barbara, Calif. 93105.

+ Nylon pressure tubing # 1207 type T Standard Wall AWG Size 18 (0.042 I.D. x 0.074 O.D.) Available from: Cadillac Plastic and Chemical Co., 313 Corey Way, San Francisco, California 94089.

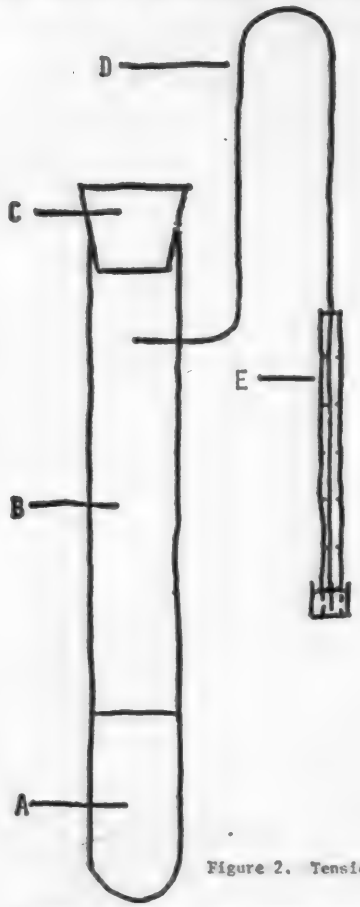
Figure 1. Soil-Water Solution Probe Construction.

App. VI-43

The suction probes need not protrude above the soil surface if they can be tightly sealed so that water cannot enter the barrel. A good general rule is that if they are to be buried, the top of the barrel should be below the saturated soil zone in order to prevent leakage into the barrel should a slight leak develop at the stopper. For this reason, it is usually safer to have them protrude above the soil surface at least 15 cm so that water that is ponded on the surface does not cover the stopper. They are also easier to service should it be necessary. On the other hand, when they protrude above ground surface, maintenance of the plot, viz-a-viz growth of plants, must be done manually.

B. Tensiometers. The tensiometers can be constructed in exactly the same manner as the suction probes except that a clear piece of lucite or glass should be glued to the top of the pipe to provide visual inspection for air leaks. If the tensiometers are buried, this features can be eliminated. A small bore nylon tube is attached to the PVC tube to provide a water connection between the PVC

tube and the mercury manometer at the edge of the plot. Commercial tensiometers using gauges may be used in place of the nylon tubing and mercury manometer. Since the main purpose of the tensiometers is to provide an indication of the moisture status of the profile, the latter may prove more convenient although more expensive. If the tensiometer is below ground surface, 2 nylon tubes will be required. (See Figure 2.)



A. Porous cup* secured with epoxy cement to barrel.

B. 1.9-cm diameter plastic polyvinylchloride barrel (Schedule 80).

C. Neoprene stopper.

D. Small-diameter, flexible, water-filled nylon tube† epoxied to PVC barrel connects the water in the barrel to a mercury reservior.

E. The nylon tube was passed vertically through a glass tube which was affixed atop a meter stick into the mercury reservoir, allowing convenient readings of the mercury length.

For additional information see References 5 and 6.

*Porous ceramic cup (1 bar) Catalog # 2131 Available from: Soil Moisture Equipment Co., P. O. Box 30025, Santa Barbara, Calif. 93105.

†Nylon pressure tubing # 1207 type T Standard Wall AWG Size 18 (0.042 I.D. x 0.074 O.D.) Available from: Cadillac Plastic and Chemical Co., 313 Corey Way, San Francisco, California 94089.

Figure 2. Tensiometer Construction.

App. VI-45

Tensiometers and suction probes should be installed so that they are at least 30 cm apart and staggered in depth so that adjacent probes and tensiometers are not at the same depth, thereby preventing interference.

One tensiometer at each depth is sufficient although 2 are recommended to preclude loss of information due to instrument failure and provide a better estimate of the soil water suction. Since it is useful to know the direction of the gradient for water flow in the profile, at least 2 depths of installation are required. Since the shallower depths are likely to undergo wider fluctuations in soil water suction, it is recommended that tensiometers be placed at the same depths as the suction probes.

V. *Test Procedure.* A. Before application of the compound, the plots should be flooded with water to a depth of 6 cm and maintained in this condition to wet the profile. This will provide a period in which to close leaks and test the instruments. At the end of this period the plots should be allowed to drain until the surface is dry enough to work.

B. *Excessive irrigation plots.* 1. To minimize the short-term leaching period, a continuous leaching procedure is recommended. Following the application of the pesticide, water is continuously ponded on the plot until 30 in. of water has entered the profile. If the pesticide is applied in the first irrigation water increment, the pesticide water should enter the soil completely before additional water is applied to continue the leaching procedure.

Sampling of the soil solution at the shallow depths should begin soon after leaching is started. Sampling should continue every 2 hr for the first 8 hr and less frequently thereafter. Some indication as to when sampling should begin at each successive depth can be ascertained from changes in the soil suction readings on the tensiometers. When the suction readings begin to decrease, this is an indication that water has penetrated to that depth and sampling should commence.

After all water has entered the plot and redistribution begins in the soil profile, occasional sampling for 2 to 3 days would provide information on pesticide movement under unsaturated conditions. When the soil profile is sufficiently dry, but still moist, soil samples could be taken as a final check on the pattern of pesticide distribution.

2. As an alternative to the above procedure, the leaching water could be applied in increments of an equivalent depth of 4 in. on the soil surface with approximately 2–5 days of redistribution between increments. This procedure might be more convenient for some investigators. Some modification in the sampling procedure might be followed although, in general, it would be much the same as that described.

C. *Normal irrigation plots.* The same procedures as above would apply to the normal irrigation or simulated rainfall treatment.

D. *Normal rainfall plots.* Normal rainfall treatments would require that sampling of the soil solution would be possible only when sufficient rainfall occurred to wet the soil profile. This treatment could logically be eliminated from a strictly leaching investigation since the time-span is such that much of the pesticide would disappear through other processes. Since rainfall is likely to be intermittent, depth of penetration could be measured by soil sampling and suction probes could be eliminated.

E. *Temperature.* 1. Temperature devices to allow continuous monitoring of the soil temperature at 15 cm and 45 cm should be installed. The temperature measurement should be made in one plot of normal rainfall and one plot of applied water. Inexpensive clock-driven thermographs are suitable for this purpose.

2. Meteorological measurements, such as air temperature and rainfall, should also be obtained in the vicinity of the test area.

F. *Specific comments.* The purposes of specifying 2 types of test leaching procedures are as follows:

1. Normal rainfall will provide information on the depth of penetration for areas where rainfall is the only source of water. Montoring of the soil solution by suction probes provides a measure of the penetration during the flow process, a condition which intermittent soil sampling will not provide, particularly when soils are too wet to sample otherwise.

2. The time factor following application will provide a comparison with the treatment involving immediate leaching.

3. The applied water treatment provides a measure of the immediate leachability of the chemical under sustained water applications. This treatment is extreme, but it provides the limit of the chemical's mobility. The 2 tests therefore provide broad limits between which the normal use and environmental conditions are likely to occur. Should the chemical move excessively under the applied water treatment, then other factors affecting the degradation of the compound and lesser leaching conditions can be considered in evaluating the actual leaching that is likely to occur under "normal" conditions. The condition of a moist profile and intermittent water application provides a favorable test condition for compounds that are either less prone to move or more prone to move depending upon the soil moisture conditions. Also the mechanics of performing the test are easier to execute.

4. The initial pesticide application is higher than normal use but not excessive. Considering other modes of dissipation, repeated applications of some chemicals and so forth, this represents a compromise in the test procedure.

5. Temperature and rainfall data are useful since these 2 factors appear to be important variables in the dissipation of pesticides in the environment.

6. Both soil solution (probe) and soil samples are included in the requirement: the former to assess the mobility; the latter to provide data that may not be evident from solution sampling.

7. A chloride salt such as KCl at the rate of 2 tons/acre could be uniformly spread on the plot surface in a dry powder form at at the time the pesticide is applied. This relatively inert tracer would provide a measure of the depth that a mobile constituent will penetrate under natural rainfall as well as under applied water conditions and a useful comparison with the less mobile pesticides.

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VOLATILIZATION STUDIES

Pesticides may be lost from treated areas by volatilization. The rate at which a substance passes from the solid phase to the vapor phase depends on a number of factors. Under environmental conditions, the effects of particle size, climate, interactions with environmental substrates or with other ingredients of the formulation must all be considered insofar as they modify the rate of loss of the pure material from a surface when the molecules diffuse through still air layers.

Vapor pressure is one of the most important factors governing the tendency of a compound to volatilize; it provides an indication of the relative tendency of pure substances to vaporize in an unperturbed situation. The rate of evaporation of a number of compounds is related to the vapor pressure p, by the relationship:

$$p = k\ (\text{evaporation rate})^n \qquad (1)$$

where k and n are parameters that depend on such factors as temperature and wind speed (*1*). Other factors influencing volatility have recently been discussed by Phillips (*2*). Field studies have indicated that the best indication of the extent of volatilization of a pesticide from soil may be gained by measurements of the instantaneous flux of water vapor over the field (*3*).

Volatilization from aqueous systems depends not only on the vapor pressure of the compound but also on its solubility. The problem is complicated in soils since adsorption occurs. However, quantitative estimates of relative volatility can be obtained from the measurement of vapor pressure, water solubility and soil adsorption.

Elaborate laboratory or field experiments are of questionable value at the present time, since techniques of measurement of volatilization rates in the field are not sufficiently developed for critical or comparative evaluation. However, it seems reasonable that careful measurements of the basic data should provide a rational basis for the assessment of environmental hazard.

Vapor Pressure Measurement—Methods. Several methods have been described in the literature for measurement of vapor pressure (*4–6*). The methods for measuring the vapor pressure of pesticides should generally be applicable to the measurement of pressures less than 10^{-3} mm is the vicinity of 25°C. Methods based on extrapolation from measurements at elevated temperatures give unreliable results, especially if the curve is extrapolated through a temperature at which a change of phase occurs.

Two principal methods are available. The first is the Knudsen effusion method described by Hamaker (*5*) in which the rate of loss of a vapor escaping through a hole of known diameter is measured. Alternatively, the jet recoil from the stream of effusing gas may be measured by a torsion balance. The second method, originally due to Regnault, is often termed the transpiration method (*6*). A stream of gas is saturated with a vapor and the vapor is collected by trapping. Measurement of the amount of material transported by a known amount of carrier gas is used to calculate the vapor pressure. This method was developed by Spencer and his coworkers (*7–9*) and used to measure vapor pressure of a number of pesticides. The changes in vapor pressure caused by adsorption on soil were also studied (*10*). The principal advantage of the latter method over the effusion method is primarily that the presence of impurities does not affect the accuracy of the results, if the method of analysis is specific for the compound under study. In both methods it is essential that the gas be saturated with vapor.

Comparison of results in the literature shows a wide range of values given for a number of individual pesticides. These vary with the technique used. It is therefore important to make an initial check of the performance of the apparatus by the use of compounds of known vapor pressure.

Estimates of Relative Volatility—Evaporation of a pesticide is probably proportional to the evaporation of water from a field. This concept was proposed by Hartley (*11*) and is based on the assumptions that the mechanisms (diffusion, convection, etc.) promoting the volatilization of water will influence other compounds equally and that the rate of volatilization of other compounds, like that of water, will be controlled by environmental factors.

The first factor is that the vapor pressure (or vapor density) of the compound will govern the amount of vapor present in the system. A second factor is the rate of diffusion of the compound through the air, which will be approximately proportional to the square root of the molecpular weight. The third factor is the weight of material carried by the diffusing molecular, which is directly proportional to the molecular weight, *M*. Equation 2 combines these factors:

$$\frac{\text{Wgt. evap. (pest.)}}{\text{Wgt. evap. } (H_2O)} = \frac{(pM^{1/2})\ \text{pest.}}{(pM^{1/2})\ H_2O} \qquad (2)$$

Hartley's calculations based on a water loss of 6 tons water/acre/day gave, for the weight of pesticide evaporated in pounds/acre/day (W), the formula:

$$W = 322.6\ (pM^{1/2})\ \text{pest.} \qquad (3)$$

This formula is approximate and does not take into account the adsorption on soils, but it is useful for the comparison of the volatility of one pesticide with another. Such calculations give a guide to pesticide behavior; for example, DDT is estimated to volatilize at the rate of 0.4 lb/acre/yr, whereas parathion would volatilize at 0.21 lb/acre/day at application rates of 0.25 to 0.5 lb/acre. However, many factors, such as adsorption, interaction with other compounds, and distribution, reduce the rate of volatilization significantly. Caution is necessary in using such predictions.

The comparison of pesticide behavior based on vapor pressure measurements may be extended to yield estimates of volatility from water or volatility from moist soil. In the former case the solubility in water is required and in the latter, the adsorption coefficient is necessary for the calculation.

Volatility—From Water. The use of water solubility to calculate volatility from water solution depends on the observation that the saturated solution should have the same vapor pressure as the pure chemical. Thus, the vapor pressure of the chemical can be used to calculate the ratio between the concentration in water and that in the air. The following formula (*12*) can be used:

$$\frac{C_{H_2O}}{C_{air}} = \frac{\text{Solubility} \times 82.06 \times 760 \times T}{pM \times 10^6} \qquad (4)$$

where C_{H_2O} is the pesticide concentration in water (ppm); C_{air} is the pesticide concentration in air ($\mu g/cc$); 82.06=gas constant (cc atm/deg·mol); 760=mm/atm; T=absolute temperature, °K (i.e., °C+273.15); p is the vapor pressure; and M is the molecular weight of the compound. Pesticide solubility is reported as ppm. This formula may not be exact, since the vapor pressure of the pure chemical may be changed because of water that dissolves in the chemical. Also, there may be some change in distribution ratio as the concentration of chemical decreases from its saturation value. However, inaccuracies are very small since most pesticides have low water solubility and, hence, a very small affinity for water.

TABLE 1.—*Estimates of volatility of chemicals from water*

[From: references 12 to 15]

Chemical	Vapor pressure [a] (millimeters per hectogram)	Water solubility [a] (parts per million)	Distribution ratio water/air (parts per million or micrograms per cubic centimeter)	Estimated practical volatility behavior from aqueous systems
Methyl bromide	1.34×10^{3}	16,000	2.30	Very volatile fumigant.
Chloropicrin	2×10^{1}	1,950	11	Volatile fumigant.
Ethylene dibromide	$.77\times10^{1}$	3,370	43.3	Do.
Dibromochloropropane	5.8×10^{-1}	1,230	164	Fumigant.
Trifluralin	[b] 1×10^{-4}	[b] .58	322	Significant volatility.
DDT	1.9×10^{-7}	.0012	326	Volatile from water.
Nitrapyrin [c]	2.8×10^{-6}	[b] 40	1,150	Volatile if surface-applied to moist soil.
EPTC	[b] 1.97×10^{-2}	370	1,844	Do.
Lindane	3.2×10^{-5}	10	2×10^{4}	Slight volatility.
Parathion	3.8×10^{-6}	20	3.36×10^{4}	Volatile from leaf surfaces.
Chloropham	1×10^{-6}	88	8×10^{4}	Low volatility.
Atrazine	3.9×10^{-7}	33	3.2×10^{5}	Nonvolatile.
Phenylmercuric acetate	9×10^{-6}	4,700	2.88×10^{7}	Do.
Monuron	5×10^{-7}	230	4.3×10^{5}	Do.

[a] Vapor pressure and water solubility at the same temperature, usually 25° C.
[b] Measurements made at the Walnut Creek Laboratory of the Dow Chemical Co.
[c] Nitrapyrin is 2-chloro-6-(trichloromethyl) pyridine.

This calculation as applied to a group of pesticides is shown as Table 1. The chemicals are listed in order of increasing value for the calculated distribution ratios, which, as the last column indicates, is qualitatively consistent with volatility from aqueous sys-

tems. The following rating system is suggested:

<100—Rapidly lost from water surfaces. If soil adsorption is low, can act like a fumigant, i.e., moves as vapor through moist soil.
10^2–10^3—Volatile from water surface.
10^3–10^5—Slight volatility from water surface.
>10^5—Nonvolatile.

Note that the vapor pressures themselves do not form a sequence, and that DDT is the most out of line. Because of its very low solubility, it is much more volatile from water than its vapor pressure would suggest. In such cases co-distillation may also affect the loss.

From Moist Soil. Volatilization from a moist soil surface is volatilization from water modified by soil adsorption. Adsorption will diminish the tendency of the chemical to volatilize. The distribution ratio between wet soil and air, if the pesticide concentration is on a dry soil-weight basis, can be calculated (*12*) by Equation 5.

$$\frac{C_{ws}}{C_{air}}=C_{H_2O}/C_{air}\ (1/r+K_d) \qquad (5)$$

where C_{ws} is the pesticide concentration in wet soil (ppm, on a dry soil weight basis); C_{H_2O}/C_{air} is the water/air distribution ratio; r is the weight of soil/wt. of H_2O; and K_d is the concentration in soil/conc. in H_2O (linear adsorption coefficient).

If the soil concentration is expressed on the basis of the total weight of soil and water, the following formula (*12*) applies:

$$\frac{C_{ws}}{C_{air}}=\frac{C_{H_2O}}{C_{air}}\left(\frac{1}{r+1}+\frac{rK_d}{r+1}\right) \qquad (6)$$

The first formula (Equation 5) was applied to a number of pesticides with r assigned a value of 6 as a reasonable moisture content. The results are given in Table 2 with the pesticides arranged in order of increasing soil/air distribution ratio, corresponding to decreasing volatility. Note that adsorption significantly reduce the volatility of parathion, DDT, and EPTC. The practical significance of volatilization must clearly include consideration of degradation and leaching as alternative pathways for loss from soil.

TABLE 2.—*Estimated tendency of chemicals to volatilize from water and from moist soil*

[From: references 12 to 15]

Chemical	Vapor pressure (millimeters per hectogram)	Solubility in water (parts per million)	Water/air concentration ratio [a]	K_d for 2 percent organic carbon [b]	Water + Soil / Air concentration ratio [c]
Fumigants:					
Cis-1,3-D	2.5×10^{3}	2,700	17.7	0.51	1.2×10^{3}
Trans-1,3-D	1.85×10^{3}	2,800	24.9	.56	1.81×10^{3}
EDB	$.77\times10^{3}$	3,370	[illegible]	.65	3.54×10^{3}
DBCP	5.8×10^{-4}	1,230	167	2.58	4.59×10^{3}
Volatile from soil:					
Chloroneb	3×10^{-3}	[illegible]	2.35×10^{3}	23.2	5.49×10^{3}
EPTC	1.97×10^{-2}	370	1.84×10^{3}	[illegible]	1.07×10^{4}
Dichlobenil	5.5×10^{-4}	[illegible]	3.48×10^{3}	[illegible]	1.2×10^{4}
Slightly volatile from soil:					
Disulfoton	1.8×10^{-4}	[illegible]	5.55×10^{3}	42.6	2.37×10^{4}
Lindane	3.2×10^{-5}	10	1.96×10^{4}	[illegible]	5.29×10^{4}
DDT	1.9×10^{-7}	1.2×10^{-2}	3.26×10^{3}	4,860	1.58×10^{3}
Nonvolatile from soil:					
Parathion	3.8×10^{-5}	20	3.3×10^{4}	[illegible]	6.9×10^{4}
Chlorpropham	1×10^{-5}	[illegible]	8×10^{3}	11.8	1×10^{5}
Atrazine	8.9×10^{-7}	[illegible]	3.2×10^{3}	3.44	1.2×10^{5}
Monuron	5×10^{-7}	230	4.2×10^{3}	[illegible]	7.67×10^{5}

See equation below:

[a] $\frac{\text{Water+soil}}{\text{air}}=\frac{C_{H_2O}}{C_{air}}\left(\frac{1}{\text{wt. (soil/water)}}+K_d\right)$

[b] This K_d represents 0.02 (K_{oc}), i.e., the distribution coefficient corrected to a "standard" soil containing 2 percent organic carbon.

[c] Wt. (soil/water)=6: volume (water/air)=1.

Actual volatility from a specific soil situation will depend upon such a variety of circumstances that it will be very variable. Spencer and Cliath (*16*) have shown, for example, that the rate of volatilization depends upon the moisture conditions at the soil surface. Since the absorption on a dry soil surface is much higher than on moist soil, a surface layer of dry soil will act as a barrier to volatilization by adsorbing the chemical. It would appear that, under field conditions, volatilization from soil may occur in "bursts" after rainfall.

The observed volatility will also depend upon the depth to which the chemical is incorporated into the soil. Thus, any attempt to measure the volatility from soil directly will necessarily be arbitrary and probably will not provide much more information than an estimate of tendency to volatilize based upon the calculated (soil+water)/air concentration ratio.

Transpiration (Gas-Saturation) Method of Measuring Pesticide Vapor Pressure. Vapor pressure of pesticides, less than approximately 0.01 mm Hg, should be determined by the gas-saturation method in which an inert gas, such as nitrogen, is passed through pesticide-treated sand slowly enough to insure equilibrium vapor saturation (*8, 10, 17–19*). The pesticide can be removed from the carrier gas stream in a suitable liquid trapping agent such as hexane (*18*), ethylene glycol (*16*), or a suitable solid trapping agent, such as Florisil (*20*) or Chromosorb 102 (*21*). The pesticide content of the trapping solution or solid-phase material is then determined with a gas-liquid chromatograph or other suitable instrument for detecting low levels of pesticides.

Apparatus. The apparatus described below can be adapted for measuring vapor pressures of pesticides in various soils by incorporating a humidity control and measuring device in the flow system in order to maintain relatively constant soil-water contents during the measuring period (*18*).

1. Constant temperature, water bath or incubator.

2. The flow system should allow for (a) careful control of air flow, for example by needle valves, to flow rates of 1 ml/min; (b) careful measurement of air flow; and (c) correction of the flows due to pressure and temperature differences between the flow meter and the saturator.

3. The saturator or the volatilization tube containing the pesticide-treated sand should be sufficiently large to allow a residence time of approximately 30 min after inert gas injection to insure equilibrium vapor saturation. This would require a volatilization tube of approximately 100-ml volume at a flow rate of 3 ml/min. For this purpose a glass tube, 3-cm diameter by 15 cm long, with ground glass joints would be sufficient. Larger saturators are satisfactory.

4. The trapping system can utilize solid-phase or liquid-phase trapping agents. Solid-phase trapping agents can be connected directly to the volatilization tube through ground glass joints or Teflon tubing. Liquid trapping agents should be contained in gas-washing bottles or similar tubes containing fritted bubblers to facilitate pesticide trapping in the liquid solvent. These can be connected to the volatilization tube with Teflon tubes or ground glass joints.

Procedures. The vaporization tube saturator should be filled with white silica sand coated with the pesticide. Quantities of relatively pure pesticide, equivalent to 1–2 percent of the chemical, should be added to the white silica sand in a suitable solvent. (The white sand should be relatively free of iron and aluminum oxides; water-washing the sand by pouring off the supernatant liquid containing the fine fraction is a desirable precaution.) After evaporating the solvent in the open air or in a rotary evaporator, the sand-pesticide mixture should be mixed thoroughly and added to the vaporization tubes. For 100-ml vaporization tubes, approximately 150 g sand should be treated with the pesticide contained in 5 to 10 ml of solvent. After connecting the vapor trap and bringing the system to constant temperature, dry nitrogen gas is passed through the saturation at flow rates of about 3 to 5 ml/min until sufficient pesticide has been trapped for analysis by a suitable analytical technique—preferably by GLC. Vapor pressure should be determined at 3 temperatures (25°, 35°, and 45° C) to allow calculation of the vapor pressure-temperature coefficient, or heat of vaporization. Vapor pressure can be measured on the *same column at various temperatures* by varying the temperature in the constant temperature water bath or incubator. Several runs should be made at each temperature on the same column, preferably at more than one flow rate within a range of 3 to 8 ml/min to ensure vapor saturation.

Flow rates should be monitored to establish the total volume of gas flowing through the saturator during each run. To calculate vapor density in weight per unit volume of gas passing through the saturator, the measured volumes are corrected for pressure and temperature differences and for the net volume of trapping solvent added to the nitrogen gas between the saturator and the flow meter. These corrections are much simpler if a solid-phase adsorbent is used since it is then unnecessary to correct for the volume for the volatilized solvent. Vapor pressure is calculated from vapor density as follows:

$$p=d\ (RT/M) \qquad (7)$$

where p is vapor pressure in mm of Hg; d is vapor density in g/1; R is the molar gas constant (62.4 when pressure is in mm Hg and volume is in liters); T is the absolute temperature; and M is the molecular weight of the pesticide.

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LABORATORY SOIL METABOLISM STUDIES

Three main types of transformation are reported to occur in soil—photochemical, chemical and microbiological.

Although photodecomposition of pesticides in air and water is a very common occurrence, little photodecomposition in soil would be expected since radiant energy is strongly sorbed by soil and therefore relatively unavailable for photochemical degradation. Nonetheless, for purposes of registration, the registrant is encouraged to conduct laboratory-scale soil degradation experiments in the dark so as to eliminate photolysis as a possible significant factor in these experiments.

Chemical transformations in soil, mediated through water functioning as a reaction medium, a reactant, or both, are widespread phenomena. The chemical reactions that occur in soil may be catalyzed by clay surfaces, metal oxides, metal ions, organic surfaces, and organic materials that can be separated from the soil. Additionally, extracellular enzymes which are widely distributed and stabilized in soil, play a significant role in the degradation of many pesticides and represent the transition point between chemical and intracellular microbiological breakdown.

Microbiological degradation is a critical factor affecting the fate and behavior of pesticides in soil (*1,2*). Whether or not a chemical is persistent, short-lived, mobile, stationary, sorbed, activated, inactivated, or eventually constitutes a residue problem may depend upon its metabolism by soil microorganisms.

Comparison of autoclaved with nonsterile soils should not in itself be considered adequate criteria for substantiating microbial degradation as the sole mechanism of pesticide dissipation. Because of the unreliable effects of soil sterilization methods on pesticide behavior and product formation in soil (*3*) and their negligible value in assessing the microbial involvement in pesticide metabolism, "sterile controls" have no value in soil dissipation protocols.

Other methods of eliminating, controlling, or reducing microbiological activity should be employed to accurately assess the contribution of the soil microbial population. For purposes of registration, it would seem most expeditious to delineate the pathway(s) and rate of dissipation from soil and to identify or characterize the intermediate products involved insofar as reasonably possible. The exact mechanism (microbial versus chemical) whereby a given pesticide degrades should be considered of more academic than practical significance.

Soil dissipation studies require the use of radiolabeled pesticides and should be designed to:

1. Characterize the rate of breakdown of the parent pesticide and products so that their stability in soil can be classified and related to reference compounds.

2. Determine products and degradation pathways.

3. Develop some concept of variability in breakdown rate as related to soil and climatic factors.

4. Determine the influence of soil and climatic factors on accumulation of different soil products.

5. Maintain a balance sheet of radioactivity applied to the experimental sample.

Protocol for Aerobic Soil Dissipation Studies—Incubation System. Two types of systems (1 open and 1 closed) can be recommended. Each has its inherent advantages and disadvantages. An open system employing a multipurpose manifold assembly has been described by Parr and Smith (*4*) for use in evaluating microbiological interactions with pesticides. (See Figure 1.) The principal advantage of the system is that the investigator can conduct soil respiration experiments under more precisely defined conditions; i.e., a relatively constant level of oxygen can be maintained in the decomposition chambers throughout incubation and the effect of different gaseous environments (involving mixtures of oxygen, nitrogen, and carbon dioxide) on soil microbial activity can be studied. Traps can also be incorporated for volatile products. An obvious drawback of this system is its complexity for large multiflask experiments.

The "biometer" flask approach (closed system) of Bartha and Pramer (*5*) has received wide usage in pesticide soil dissipation studies. Other closed systems containing the basic elements illustrated in Figure 2 may be equally acceptable. Inclusion of a pure oxygen inlet in this system allows the replenishment of oxygen used by the soil on a one-to-one basis. If air is substituted for oxygen, the flask atmosphere becomes progressively enriched in nitrogen and less in oxygen, thus ultimately creating more nearly anaerobic conditions.

Soil—Soil dried to a workable moisture content should be screened through a 2-mm screen before weighing into incubation flasks. An adequate ratio of head space versus soil volume should be maintained. Fifty g of soil appear adequate for 250-ml biometer flasks. Moisture content should be determined so that samples can be adjusted to correct moisture level after treatment.

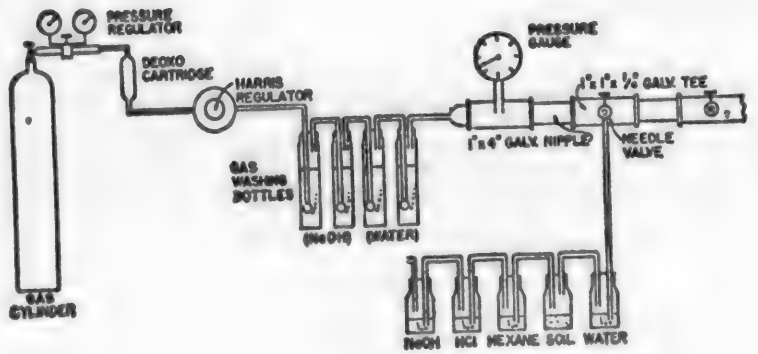
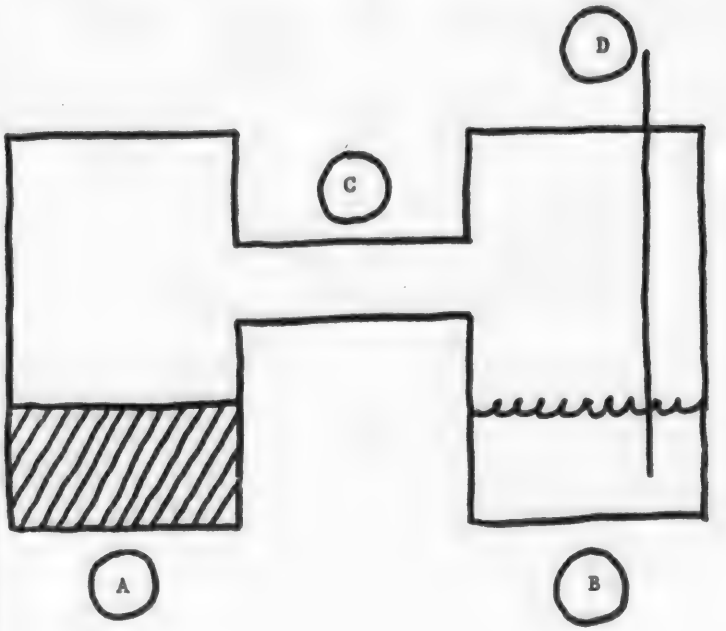


Figure 1. A Schematic Diagram of Multi-Purpose Manifold Assembly For Supplying Uniform and Constant Aeration to Individual Soil Systems During Incubation. (From Ref. 4.)



A Soil Flask

B NaOH Sump

C Diffusion Arm

D Oxygan Inlet Tube

Figure 2. Schematic Diagram of Soil Incubation Flask.

Soil Treatment. Soil pesticide treatments are preferably applied in sufficient aqueous solution to bring the soil to its desired moisture level and incorporate the pesticide simultaneously. Insoluble pesticides may be applied in acetone or ethanol. A maximum of 1-ml organic solvent per 100 g of soil should be used. The soil should be thoroughly mixed and watered to the desired level; the recommended soil moisture content is 75 percent of 0.33 bar moisture.

Replications. Either 2 or 3 replications are required.

Incubation Temperatures. A single temperature of 25°C is recommended for metabolism studies. A minimum of 2 temperatures are recommended for rate studies.

Chemical Purity. The chemical should be of known purity, i.e., technical or reagent grade. Radioactive labeling should be in a position of the molecule which allows characterization of significant breakdown products.

Carbon Dioxide Evolution. KOH or NaOH is used as a carbon dioxide trapping solution. The solution is sampled at appropriate intervals. The carbon 14 content of the trapping solution can be determined by liquid scintillation. Care should be taken to distinguish between radioactive carbon dioxide and other volatile carbon 14 products in the trapping solution.

Incubation Period. Sufficient units should be established to permit periodic sacrificing and characterization of degradation rates and product appearance and disappearance. Incubation should continue to a 90-percent loss of the parent pesticide and until patterns of accumulation and decline of products are established. Maximum length should be no more than 1 year since incubations longer than a single growing season probably become misleading due to the equilibrium established in the constant incubation conditions of a laboratory study.

Product Extraction and Identification. Incubated soils should be exhaustively extracted by a sequence of solvents. Product identification should be conducted on all materials comprising more than 10 percent of the initial application, or 0.1 ppm, whichever is greater. Other products in excess of 0.01 ppm should be identified where feasible. Characterization of unidentifiable products may include TLC mobility, GC retention time, relative polarity, UV absorbance on TLC, radioactive content (percent of original or total), or possible TLC spray reactions. In addition, extraction *p*-values are useful. These are defined (*6,7*) as the numerical fraction of solute (product of interest) partioning into the upper phase of an equivolume, two-phase immiscible solvent system. For many compounds or relatively low polarity, the binary system composed of hexane and acetonitri e is desirable. However, for most polar products, other systems are more appropriate. When possible, a solvent system should be selected to give the greatest numerical spread between different products with similar physical properties. The utilization of this technique for polar organophosphate pesticides is described by Ahuja (*8*). When more than 10 percent of the radioactivity from the initial application remains in the soil after exhaustive extraction, the registrant shall proceed with a soil bound residue analysis.

Data Interpretation. The stability of parent material shall be reported as the average time required for a 50-percent loss ± standard deviation from the study. If products also warrant separate studies, they should be reported in the same manner.

Protocol for Anaerobic Soil Dissipation. Flooding or waterlogging a well-aerated soil can have an effect on many oxidation-reduction systems, which, in turn, may indirectly affect pesticide metabolism, fate and environmental behavior. For a more detailed discussion of soil anaerobicity, refer to the literature (*1–5, 9–13*). Two methods have been utilized to attain anaerobicity: (1) The atmosphere of an incubation chamber is purged of oxygen by substitution of inert gases carefully purified of their oxygen; or (2) a sample is waterlogged to decrease rate of oxygen diffusion below that required by the microbial pressure within that sample. Because some inert gases are toxic to some microbial populations, the second method would be preferred.

Establishment of Anaerobicity. The recommended method for establishing anaerobicity is flooding, with sequential evacuation and replacement of the atmosphere with nitrogen. The thickness of the water layer over the soil should be 2–3 cm. After flooding, the soil container should be evacuated and refilled with nitrogen 2 to 3 times to assure complete removal of oxygen. Substitution of inert gases without a water barrier to establish anaerobiosis is not recommended. A water barrier protects against low level oxygen leakage that is bound to occur with any but the most leakproof type of apparatus suitable for high vacuum work; rubber and most organic synthetics, for example, are too permeable to oxygen.

Incubation Vessel. The incubation vessel should be sealed with a cap; a closed container minimizes volatilization effects. Inlets and outlets should be provided to permit evacuation and introduction of nitrogen. All-glass systems are preferable; if unavailable, a bottle with a tight-fitting cap may be substituted.

Soil Types. The soil used for anaerobic studies should be the same soil used for detailed aerobic soil metabolism, except for specific anticipated uses, such as rice soils or aquatic pesticides. Preincubation of soil to assure anaerobic metabolism is unnecessary, if the procedure outlined above is followed.

Soil Treatment. The pesticide mixed with water is the preferred application. If solubility does not permit, then acetone or methanol diluted in water may be used. Pesticide application should not exceed 1 ml organic solvent per 100 g soil. Treatment concentrations recommended are 1 and 10 ppm for comparative rates of metabolism. Detailed product studies should be conducted at the 1-ppm level. Incubation should be at 25°C. and in the dark.

Methodology. Additional procedures to be observed in the anaerobic studies include the following.

1. Sampling schedule used in anaerobic studies should parallel that used in aerobic studies. Anaerobic experiments should be conducted to the point where degradation rate can be established or interpreted.

2. Products different from those observed under aerobic conditions should be submitted to aerobic investigations.

3. Product identification and characterization should extend to same level as that in aerobic dissipation.

4. Both soil and water should be analyzed for carbon 14 content at each sampling period. Carbon dioxide may be monitored if desired. Carbon-14 balance procedures are encouraged; however, losses may be incurred through carbon 14 methane or acidic gas production.

5. The initial extraction solvent should be hexane to trap any possible methane. More polar solvents should follow. Hexane should be examined by gas-liquid chromatographic procedures sensitive to methane or other similar products.

6. A gas chromatography-gas proportional counting system capable of simultaneous measurement of metabolic gases (hydrogen, methane and carbon dioxide) from methanogenic systems has been described by Nelson and Zeikus (14).

7. Data should be reported in terms of disappearance of the parent pesticide and the formation and disappearance of products, with respect to length of incubation.

8. When more than 10 percent of the radioactivity from the initial application remains in the soil after exhaustive extraction the registrant shall proceed with a soil bound residue analysis.

Extraction Procedures for Soil Bound Residues. Often during soil dissipation experiments, a certain amount of radioactivity remains unextractable and is referred to as "bound residue." A proposed interim definition of soil bound residues is "that unextractable and chemically unidentifiable pesticide residue remaining in fulvic acid, humic acid, and humin fractions after exhaustive sequential extraction with nonpolar organic and polar solvents". Although the importance of these residues is not fully understood at present, a significant amount of many pesticides residues in soil fall into this category. Thus, it is essential to characterize the distribution of such residues in the soil organic matter matrix.

Stevenson (*15*) has suggested that methods used to fractionate soil organic matter can be placed in 2 categories: (1) Those used to fractionate and estimate compounds characteristic of plant tissues; and (2) those based on the division of the humus into subclasses possessing similar solubility characteristics.

The great difficulty with all fractionation procedures is that the methods employed either separate out products which are not definite chemical entities or that fractionation methods may form artifacts which do not have the properties of the original material. Nevertheless, the various fractionation procedures have proved useful for studying soil organic matter and soil bound pesticide residues.

Before determination of the bound residues, the soil should be exhaustively extracted with nonpolar and polar solvents, such as ether, benzene, ethyl acetate, acetonitrile, water, etc. It is possible that, in certain cases, a mild extraction of soil with dilute alkali (0.1 *N* KOH or NaOH or NH_4OH) after the exhaustive extraction with nonpolar and polar solvents may release some identifiable unbound degradation products. For characterization of bound residues the classical method of fractionating humus is based on the extraction of organic matrix from soil with caustic alkali and the further subdivision of the extracted material by partial precipitation with mineral acids.

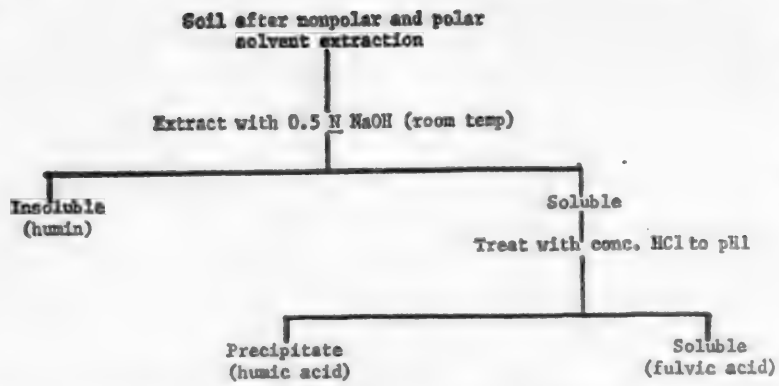

Soil after nonpolar and polar solvent extraction
Extract with 0.5 N NaOH (room temp)
Insoluble (humin)
Soluble
Treat with conc. HCl to pH1
Precipitate (humic acid)
Soluble (fulvic acid)

Figure 3. Fractionation Scheme Used to Characterize Distribution of Radiolabeled Bound Pesticide Residues in Soil.

In practice, 5 g of soil—after exhaustive organic solvent extraction—is shaken with 25 ml of 0.5 *N* NaOH in a shaker for 24 hr at 25° C. The soil solutions are then transferred to centrifuge tubes and centrifuged at 3000 rpm for 10 min. The supernatant is decanted, the soil is washed twice with 10-ml portions of 0.5 *N* NaOH and recentrifuged. The soil is finally washed with 3 10-ml volumes of water, centrifuged, air dried and set aside for combustion analysis. Unextracted radioactivity in soil solids is considered to be associated with the humin fraction and is measured by combustion analysis.

The combined supernatant and water washes are analyzed for radioactivity by liquid scintillation counting. The combined supernatant is then acidified with concentrated HCl to approximately pH 1. After the humic acids have precipitated, they are centrifuged. The remaining supernatant contains the fulvic acid fraction. The precipitated humic acids may be redissolved in 0.1 *N* NaOH. Radioactivity of the fulvic acid and redissolved humic acid fractions may be determined by liquid scintillation.

Although further characterization of these fractions will not be considered mandatory at this time, the registrant is urged to consider additional analytical methods proposed in the scientific literature (*15*).

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FIELD DISSIPATION STUDIES

Scope—Objectives. The objectives of field dissipation studies of pesticides are as follows:

1. To determine the rate of dissipation of the pesticide and its significant degradation products under fallow and cropped field conditions.

2. To determine the nature of significant degradation products of the pesticide under field conditions and compare with laboratory metabolism results.

3. To determine the potential carry-over of the pesticide and significant degradation products under field conditions for single applications and, where applicable, 3-year repeat applications.

4. To evaluate leaching in the soil under field conditions.

Two types of studies are required: (1) A qualitative study of the pattern of degradation of the pesticide under field conditions; and (2) one or 2 quantitative studies of the rates of dissipation of the pesticide and its significant degradation products under field conditions.

Qualitative Studies. Aerobic and anaerobic degradation studies of the pesticide in soil under laboratory conditions establish only the pattern of degradation of the pesticide in soil. A field study, involving application of the pesticide in the manner in which it is to be used, also takes into account the additional influence of light and/or plants on the pattern of degradation of the pesticide in the environment.

The radioactive pesticide should be applied as it would ordinarily be used with respect to method of application, type of formulation and rate of application. Where application to plants is involved, normal cultural practices should be used.

Sampling of the soil at various intervals and analysis for the pesticide and its significant degradation products should be conducted to develop a picture of the rates of disappearance of the pesticide and the rates of appearance and disappearance of its degradation products in soils. Plants should be harvested at maturity according to accepted practices. All soil and plant food and feed samples should be suitably processed for analysis and analyzed for discrete radioactive compounds and generally characterizable residues

If significant amounts of radioactive residues are found in the soil at harvest, planting and analyses of rotational crops for discrete residues may be required. Furthermore, qualitative field dissipation studies may be required on more than one crop where there is some reason to believe that different degradation products will be encountered with different crops.

Quantitative Studies. Laboratory metabolism studies in soil and qualitative field dissipation studies establish the significant degradation products of the pesticide under field conditions. The aims of the quantitative field study are to establish disappearance curves for the pesticide and its significant degradation products under field conditions (predicting carryover and potential residue buildup at the same time) and to evaluate leaching into the soil profile. Most of the applications will be to soils and to plants. Normally the experiments would run for a year. However, where significant carryover is expected, 3-yr repeat applications may be required. Attempts should be made to correlate results observed for rates of dissipation under laboratory conditions with those observed under field conditions.

Table 1 summarizes and contrasts the qualitative and quantitative studies used in field dissipation experiments. Study II represents an alternate approach to residue Study III, which is potentially useful should analytical problems arise from Study III. Because the pesticide is incorporated in fallow soil in Study II, losses from photodegradation, plant uptake, and volatilization are minimized. Study III is normally conducted as a part of crop residue studies related to tolerances.

Protocol for Field Dissipation Studies—Site Locations. Sites for the test should be located in the regions of usage. These regions are outlined in USDA Handbook 296, *Land Resource Regions and Major Land Resource Areas of the United States* (1). Each site should be located on a major agricultural soil of the region and must be available over

a 3-yr period to encompass 3-yr repeat application studies. Depending on the test, up to 4 or more sites are suggested. Descriptions of the soil profiles (obtained from the published soil survey) and heights of the water tables are required.

TABLE 1.—*Types and characteristics of field dissipation studies*

Parameter	Patterns of dissipation, study I	Rates of dissipation	
		Fallow field study, study II	Residue study, study III
Objectives [1]	1,2,4,3	1,3,4,2	1,3.
Compound:			
Form	Radioactive	Nonradioactive or radioactive.	Nonradioactive.
Formulation	Technical or most widely used formulation.	Technical or most widely used formulation.	Most widely used formulation.
Locations	1	2	4.
Plots per location	1	2	2.
Crop	Yes	No	Yes.
Rate	Normal maximum annual recommended.	Normal maximum annual recommended.	Normal maximum annual recommended.
Application method	Normal pattern	Incorporate 0 to 7.5 centimeters.	Normal pattern.
Weed control	Hand weeding	Hand weeding	Normal cultural practices.
Sampling times (soil)	Sufficient to indicate dissipation curve.	Sufficient to establish dissipation and leaching curves.	At end of crop season, later if carryover occurs.
Sampling depth	7.5-centimeter increments in 0- to 30-centimeter zone; deeper as needed.	7.5-centimeter increments in 0- to 30-centimeter zone; deeper as needed.	15-centimeter increments in 0- to 30-centimeter zone; deeper as needed.
Duration	1 crop season	12 mo (minimum) or 90-percent dissipation.	1 crop season, longer if carryover occurs.

[1] Objectives as defined in the text.

2. SCS soil survey photo maps, if available.

3. Description of area from county soil survey maps.

4. Coordinates of field plots on topographic map, if available.

Climate. A general description of the climate of each site must be given; this is obtainable from various sources, including county soil survey maps.

The specific climate during a study should also be documented. Temperature patterns should be characterized for air and soil. If soil temperature data are not obtainable, as a minimum, air temperature records from the nearest U.S. weather station can be used. For actual soil measurements, temperatures at 3, 6 and 18 in. beneath the soil surface might be considered.

Minimum information regarding moisture during an experiment is a documentation of rainfall and irrigation patterns. Rainfall should be determined directly at a site with a rain gauge. Actual soil moisture patterns are helpful and can be determined by weekly sampling or by weekly readings of soil moisture tensiometers or soil resistance blocks. These could be located at 3, 6 and 18 in. beneath the soil surface. Twenty-four hr should be allowed after irrigation or rainfall before readings are taken.

Soil. The soil comprising each experimental area should be described in terms of soil series name, series description, textural classification, and the following physical properties: organic carbon content (%); soil pH (in 0.01 *M* $CaCl_2$ suspension); 1/3-bar soil moisture percentage; sand, silt, and clay contents (%); and cation-exchange capacity. The methods used in these determinations should be described or appropriately referenced. Information on the predominant clay minerals is usually available within the published soil survey and should be reported.

Pesticide Formulation. The most widely used formulation or the technical pesticide may be applied.

Methodology. Methods are summarized in Table 1. As indicated, each study encompasses several objectives. Recommended tests for establishing a soil dissipation curve consist of fallow plots with the chemical incorporated into the top 0–7.5 cm (approximately 0 to 3 in.) after spray application (Study II). The tests for carryover of residue (Study III) should utilize other plots established primarily for collection of plant residue data; these should be located near the fallow plots. Smaller plots (Study I) are also cropped, but their main purpose is to verify the patterns, rather than the rates, of dissipation. In the past, soil dissipation studies have utilized either bare plots or plots that simulate actual use practices. When one technique is used in the absence of the other, the resulting data suffers seriously in the following manner.

Data from actual use plots generally contain significant unavoidable errors because of the presence of a cover crop. Error arises from 2 sources. The first is the uneven distribution of the chemical initially applied to the soil because of the method of application and interception by the cover crop. The second is the renewal of chemical in the soil through transfer from plant to soil during the course of the study. This renewal can occur through washoff root exudation, and incorporation of crop residue at the end of the growing season. Thus, meaningful soil dissipation curves often cannot be obtained from such data. However, an actual use plot is necessary to determine carryover because of the possible transfer of chemical from crop to soil.

On the other hand, the fallow plot technique fails to consider the movement of chemical from plant compartment to soil compartment and, thus, it may or may not be suitable for evaluating carryover. This bare plot does, however, provide meaningful data for a soil dissipation curve because of the absence of the cover crop. The method of Baude, Pease and Holt (*2*) is particularly suitable for the study of field soil dissipation with radiolabeled pesticides and is the preferred method.

Sampling. Samples for Studies I and II should be taken before application (for a supply of check soil), immediately after application (for zero-time), 4 to 5 periods between zero and fall freeze, and once before planting the following spring. Replicates analyzed separately should be obtained at some, but not all, samplings to estimate variability for each experiment. Suggested samplings for replication might include zero and last sampling before winter.

In actual use tests for studying carryover (Study III), the most important sampling periods are zero-time and the following spring prior to planting the next crop. Soil samples should be obtained at these times; sampling in the fall is also advised. Zero-time serves to show how much chemical reaches the soil initially and the following spring shows whether residues are still present. True assay of the residue level in soil is possible only after all of the plant material has been incorporated; sampling at spring preplant is the best time for this condition.

Sampling periods between these points have less value, because of the large variability in application to soil initially and the continued renewal of the soil level by transfer from plant material to soil. However, the number of sampling periods for Study III will depend on whether Study II is conducted. When Study II is conducted, the sampling intervals described will be acceptable. Otherwise, a sufficient number of samplings should be conducted to permit plotting a well defined degradation profile.

All soil samples should be taken at sufficient depth and at sufficient intervals to be all-inclusive of parent chemical and significant metabolites in order to show degradation and extent of downward movement. Increments suggested in Table 1 will adequately define the vertical distribution. Although other methods can be used, multiple soil cores provide a convenient means of obtaining samples. Care should be exercised to prevent cross-contamination between various soil horizons. A method used by several researchers which appears suitable is described by Turner (*3*). The sampling method must be statistically valid and well-described. For other discussions of soil sampling, see reference (*2, 4–9*).

Treatment. Treatment should consist of the maximum use rate stated for a use pattern. Interpolation of results can be made for lower use rates, but extrapolation to higher dosages is risky.

Repeat Applications and Subsequent Cropping. If significant residues are present in soil at the end of the season, repeat applications, rotational cropping, or both, may be required to establish that no significant accumulation of residues or carryover of residues into rotational crops occurs.

References

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PART VII

Hazard evaluation:

Human and domestic animal toxicology VII.A–1
Aquatic toxicology VII.B–1
Wildlife toxicology VII.C–1

SUBPART A

Hazard evaluation: Human and domestic animal toxicology VII.A–1
Methods for study of acute toxicity VII.A–5
Methods for study of subacute and chronic toxicity VII.A–7
Methods for study of inhalation toxicity VII.A–12
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PART VII—HAZARD EVALUATION

SUBPART A—HUMAN AND DOMESTIC ANIMAL TOXICOLOGY

Part VII of the Registration Guidelines establishes the hazard evaluation data requirements necessary for the registration of pesticides. This Subpart sets forth acceptable protocols for the development of such data by establishing the general considerations to be followed in designing a specific testing procedure for a particular pesticide and specified data requirements. The general discussion is followed by references to protocols which the agency has determined are acceptable for developing hazard evaluation data.

In designing a specific test procedure, the pesticide developer must construct a testing protocol adapted to the specific chemical nature of the pesticide and the type of exposure to man or the environment which may be anticipated from the pesticide's use. The protocol references contained in this Subpart establish minimum experimental testing standards which must be met in planning, executing, and reporting data so that the data on the pesticide's hazard may be evaluated meaningfully. Each reference was chosen by EPA as an example of an experimental protocol which meets the general standard for experimentation required by EPA. Numerous other protocols were reviewed by EPA and have not been included in this Subpart because of a determination that the protocols would not provide the meaningful, reliable data required for an accurate hazard evaluation.

Besides setting forth specific protocols, the references cited in this Subpart also provide a discussion of the fundamental factors which should be weighed for developing an adequate testing design; for example, the variables to be considered in the selection of testing species, strains, and sample size and specific aspects of oncogenic and mutagenicity toxicity studies. The pesticide developer must consider these factors in designing specific testing protocols. EPA endorses the discussions contained in the references and will be guided by those factors in evaluating the adequacy of data submitted for registration. Specifically, knowledge of the chemical nature, purity and stability of the compound to be tested, the bio-chemical reactions of specific groups, the physiological activity of similar compounds and the techniques for measuring suspected chemical and biological changes of such compounds must be considered in designing an adequate experimental testing procedure is essential. Occasionally, it may be possible to predict the physiological action of a compound by structural relationship to other compound with known physiological actions. However, such predictions based on structure similarities are not infallible and general screening procedures for possible physiological actions must be done.

SELECTION OF SPECIES

A reliable *in vitro* screening technique is not available for determining the species most closely approximating the human physiological response to many pesticides. In some cases, it is possible to obtain metabolic and kinetic data in human volunteers or by epidemiological studies of occupationally exposed individuals which can be compared with similar data from experimental animals. If such data are available, the experimental animal species most closely resembling the known human response should be selected for additional long-term studies.

In reporting each study, the species and strain, source, sex, age, weight range and all other relevant details must be clearly stated.

SELECTION OF ROUTE OF ADMINISTRATION

The basic consideration which determines the route of administration is the anticipated route of exposure to man or the environment from the use of the pesticide. The experimental exposure should be the same or as closely related as possible to that anticipated for humans. Where the anticipated exposures indicate, for example, that the gastrointestinal tract represents the route of entry into the body, principles of proper established oral dosing must be applied. In the case of agricultural pesticides, the compound should be incorporated in the diet of the test animal and not merely given by oral intubation. If more than one route of exposure is anticipated or observed, sufficient testing must be done with each route to determine the toxicity or to define the difference in toxicity.

In reporting data, the details of the method of administration must be provided; for example, incorporated into ground laboratory chow as 25 percent corn oil solution; all diets including control adjusted to a 1 percent added corn oil level; or diets available ad libitum.

END POINTS

The biological activity of a pesticide may be evidenced in a change in the appearance of an organ or tissue of the test animal (organic change) or in a change in the activity of the organ or tissue (functional change). Thus testing must not be limited to the detection of organic changes, i.e., those detected by growth curves, organ weights, hematology and gross and microscopic pathology, but must provide a total evaluation of any changes in the test animal.

METHODS FOR STUDY

Subpart A is composed of sub-sections or "Methods for Study." As outlined in the table of contents to this subpart, these Methods for Study correspond to the nine test areas requiring data for human and domestic animal toxicology. In addition to the fundamental factors, listed above, for developing an adequate testing design, each sub-section describes additional principles or procedures needed for the particular test.

ACCEPTABLE PROTOCOLS AND GENERAL REFERENCES

Following each "Methods for Study" in this Subpart references to detailed examples of presently acceptable protocols and test methods are cited. These protocols and test methods are those presently considered adequate for developing data acceptable for registration purposes. The Agency recognizes that applicants may be aware of other test protocols which are equally effective for particular purposes and that new procedures will be developed in the future. It also recognizes that the test methods described sometimes may not be well suited to the evaluation of particular products. Thus, modification of the specific procedures contained herein may be appropriate provided that the intent and reliability of the suggested methods are met. In cases where an applicant proposes to deviate from the procedures suggested, an applicant should consult with the Agency on the specific data development methods to be employed. In addition, in a particular case, the applicant or the Agency may determine that all or any portion of a referenced protocol is not adequate for hazard evaluation. Upon such determination the Agency will work with the applicant to design an appropriate protocol(s).

The general references, which also follow each "Methods for Study," deal with specific questions which must be addressed in designing a specific test protocol. These include: Selection of diet, species, and strain; number of animals; duration of exposure; proper control groups; specific clinical measurements; statistical treatment of data. The investigator is encouraged to become familiar with these articles, since they provide the current experience of the profession and are valuable in the conduct of good toxicological research.

References

Committee on Problems of Drug Safety, National Research Council. 1969. Application of metabolic data to the evaluation of drugs. *Clin. Pharmacol. Ther.* 10:607–634.

METHODS FOR STUDY OF ACUTE TOXICITY

The following information should be made available in any report of studies on the acute toxicity of a chemical agent:

1. Exact identification of the compound studied, including chemical name, structure, and purity, with quantities of known and unknown contaminants given so that 100 percent of the sample is accounted for.

2. The species and strain(s) of test animals used, including source, sex, age, weight range, diet, housing and conditioning before the time of test.

3. The method of selection and method of division of animals into test groups.

4. All doses used, including the composition of all diluents; number of animals per dose level; the route(s) of administration together with the method(s) of administration; and nutritional conditions, such as fasting and period of fasting, before test.

5. The observation period; observational methods utilized; and description of premortem and nonlethal effects.

6. The LD–50 and its confidence limits together with an evaluation of the slope of the lethality curve and a maximal nonobservable-effect dose level; and reference to the statistical methods employed.

7. When indicated, as in delayed deaths or symptomology, the inclusion of gross pathology and possibly histopathology.

The conduct of acute toxicity tests should be in accordance with currently acceptable

*More detailed *Contents* pages precede each Subpart.

principles of toxicologic expertise and statistical procedures as exemplified in the following references.

Acceptable Protocols and Methods

Hagan, E. C. 1965. Appraisal of the safety of chemicals in foods, drugs, and cosmetics—Acute toxicity. Assoc. of Food and Drug Officials of the United States. Topeka, Kansas. Pp. 17–25.

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General References

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Weil, C. S. 1972. Guidelines for experiments to predict the degree of safety of a material for man. *Toxicol. Appl. Pharmacol.* 21:194–199.

Weil, C. S., and G. J. Wright. 1967. Intra and interlaboratory comparative evaluation of a single oral test. *Toxicol. Appl. Pharmacol.* 11:378–388.

METHODS FOR STUDY OF SUBACUTE AND CHRONIC TOXICITY

In general, the subacute or chronic studies should define the biological activity of a compound and provide a reliable estimate of the lowest dosage required to produce that effect following repeated exposure.

Every study should include the selection of the proper animal species. Two general principles are available to guide the investigator in selection of these species.

1. Whenever possible, one species should permit comparison of the toxicity of the test compound with known compounds tested in the same species as well as in man. This permits indirect estimation of the toxicity of the test compound to man through other compounds. At the present time, the rat permits this comparison better than any other experimental animal for most compounds.

2. Another mammalian species, other than rodent, should be selected on the basis of its particular similarity to the human in relation to the suspected physiological and metabolic mechanisms affected by the test compound. If no valid basis for such a prediction is available, safety is best assured by selecting the second species on the basis of greatest sensitivity to acute or short-term subacute toxic effects.

Selection of Route of Administration. The selected route of administration should be as nearly identical to the potential human exposure as possible. When the test material cannot be administered to animals by the proposed route because the animal will refuse to eat the test diet, administration by stomach tube may be necessary. In most cases, however, the animal can be conditioned to the off-flavor, smell, etc., by gradually building up the dietary concentration to the desired level.

Selection of Dosage. Before the selection of dosage, a review of the acute toxicity should be made. This review should consider not only lethal dose but also the variation in animal response to the same dose and the slope of the dosage-mortality curve.

Before designing a protocol for a subacute study, it is frequently desirable to perform a dose-range finding study. As the term indicates, such a study is designed to provide a reasonable estimate of the maximum dosage an animal will tolerate on repeated administration. It is a rapid empirical procedure to eliminate inappropriate dosages before more extensive subacute and chronic experiments. The most important observation is death. Since these experiments are of a very short duration—generally 1 to 2 wk—death at any dosage level is sufficient justification to eliminate that dosage from subsequent long-term studies. Additional observations may include evidence of the acceptability of diet, loss of body weight, organ weight changes, and symptoms.

Subacute experiments should not be considered as preliminary. Properly designed and conducted, the subacute experiment will provide the majority of the basic observations upon which the decisions for acceptability of use and commercial production will be based. Even when chronic experiments are later conducted, the observations made should be an outgrowth of initial suspicions or discoveries of the subacute phase. Therefore, effort should be concentrated in the subacute experiment on providing the best available screening for deleterious changes.

One of the principal values of a chronic study is that accelerated senile, degenerative and carcinogenic changes can be detected. Adequate numbers of animals should be used to permit detection of such changes, which may occur only in a fraction of the population of test animals. The number of animals will be partially dependent on the incidence of the effect in the control population as well as the desired degree of assurance that the effect is not produced in the experimental species selected. Several specific references deal with these considerations in depth.

All quantitative measurements taken should be examined by a statistical comparison of dosed and control animals as well as statements of central tendency and variability.

Acceptable Protocols and Methods

Anonymous. 1973. The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity. Published by Minister of Health and Welfare, Canada.

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METHODS FOR STUDY OF INHALATION TOXICITY

A pesticide can be inhaled in either the vapor phase or as an aerosol, i.e., dust or mist. Inhalation may be a serious problem for consumers, applicators or field workers; the hazard may be altered by the type of formulation and mode of application. Studies designed to evaluate the potential hazard to humans must, therefore, relate to the physical form of the pesticide under specific use conditions. It should be stressed that a number of factors play roles in determining the ability of a material to be dispersed as an aerosol and, once dispersed, to remain airborne long enough to pose an inhalation hazard. It should also be recognized that inhalation exposure is often a more difficult procedure than administration by any other route. In general, consultation is advisable with scientists with experience in the use of inhalation chambers, vapor and aerosol dispersion, and sampling and analysis of chemical components present in the air of animal exposure chambers.

Inhalation, as a route of entry, results in the potential for (1) toxic effects at the site of entry, i.e., the lungs, as well as (2) the same systemic toxic effects that may occur from entry of the pesticide into the body via other routes. Specialized studies for changes in pulmonary function may be the most sensitive measurement of effect when the pesticide has its primary action on the dung itself. Systemic effects, including cancer, are measured by the same techniques utilized in studies with oral administration. Only recently have scientists begun to evaluate teratogenicity, mutagenicity and reproduction effects following inhalation exposure. No attempt is made here to document the specialized studies for pulmonary function, or the classical biochemical, physiologic and pathologic observations for systemic toxicity.

General principles and procedures for inhalation studies have been discussed by Drew and Laskin (1974) and by the Committee on Toxicology of the National Academy of Sciences-National Research Council (1964). These reports point up the necessity of considering a wide variety of factors which might have a much stronger bearing on inhalation hazard than hazard by other routes of administration. The other references are provided as representative applications of these principles to specific materials or groups of materials in a manner consistent with the state of the art. It should be stressed, however, that none of the studies can be considered comprehensive; rather they were directed to and answered questions concerning toxicity of the materials within a highly limited framework.

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METHODS FOR STUDY OF SKIN AND EYE TOXICITY

Handling any chemical or chemical formulation in quantity creates a potential for contamination of skin and eyes. Skin contact may result from accidental spillage or simple environmental contamination and can produce toxic effects by percutaneous absorption which may be similar to those resulting from ingestion or inhalation. Such contact may also produce dermatitis of the primary irritant or delayed hypersensitivity type. A variety of protocols for the study of acute and chronic percutaneous absorption and irritation are available for use with a limited number of animal species. Adequate procedures for the study of delayed hypersensitivity in animals, however, are not extensive, and the results of such animal studies cannot be considered entirely indicative of the results of exposure in humans. For this reason, protocols are available for human patch tests which are designed to determine the potential for the production of sensitization in the exposed humans. Such studies should be designed and performed by a competent dermatologist since the subjects are humans and there are medical, ethical and legal aspects to be considered.

Contact with eyes usually is the result of accidental contamination but may also result from exposures to atmospheres containing the chemical. Animals exposed for inhalation studies, where whole body exposure occurs, should be observed for both skin and eye effects. Acute eye contact studies can be made following one of several protocols; most employ the rabbit as the test animal.

Acceptable Protocols and Methods

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METHODS FOR STUDY OF REPRODUCTIVE AND TERATOSENIC TOXICITY

The physiological stresses associated with reproduction and lactation offer a unique opportunity for revealing toxic manifestations in experimental animals. The endocrinological changes associated with the reproductive cycle in the female and the anabolic systems involved in embryologic and fetal growth constitute a challenging background against which to test the toxic potentiality of a pesticide or other foreign substance. Conceivably, under these conditions, enzyme systems are called into play that are otherwise dormant, if indeed, existent. Behavioral phenomena, such as loss of sexual interest or maternal neglect, and defective prenatal developments are manifestations of toxic effects that can be detected only in appropriately planned experiments.

Abnormal reproductive performance may result from the direct influence of the toxic substance on fertility or on conceptual interest of one or both sexes, impaired implantation or gestation, prenatal undernutrition or placental transfer of toxicity to the developing embryo or fetus. Depending on the nature of the chemical and the degree of environmental or dietary exposure of man or other target species, the design of experimental protocols may extend from sin-

gle reproductive cycles to multigeneration studies.

By far the most commonly used laboratory animal for reproduction studies is the rate. However, procedures have been described employing mice, hamsters, rabbits, swine and primates. For studies involving the environmental impact of pesticides, avian species are also recommended. (See Part VII. Subpart C.)

By dosing test materials before mating, effects on both male and female fertility may be noted. Copulation is established by the appearance of sperm in the vagina and the progress of gestation and delivery are noted. Litter size and weight and growth and survival of pups during the immediate postnatal and lactation periods are recorded. Observations such as these may be quantitated and statistically evaluated.

Reproductive cycles may be continued where the duration of fertility and gestational capability are of concern. In multigeneration studies, however, observations are generally limited to 3 or 4 generations and to 2 litters per generation, one of which is delivered by caesarian section. Signs of embryo- or fetotoxicity are noted and the fetuses are examined macroscopically for teratologic defects, the soft tissues after sectioning and the skeletal structure after clearing and staining.

Teratologic screening studies have shown that the rat and mouse provide more consistent and reliable data than the rabbit or hamster. The number of matings per dosage group required for adequate statistical interpretation of responses militates against the use of larger animals in reproduction and teratologic studies. However, where species differences may exist and must be clearly defined, the use of pigs, dogs or monkeys is indicated. It should be noted, however, that interspecies variations in response and the absence of correlation with human experience raise doubts concerning the significance of teratologic tests.

The following references cover basic principles and procedures employed, as recently described by pioneer workers in the field. They include detailed protocols recommended for regulatory purposes by the U.S. Food and Drug Administration and the World Health Organization.

Acceptable Protocols and Methods

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METHODS FOR THE STUDY OF MUTAGENICITY

A primary objective of mutagen testing is to determine whether a pesticide has the potential to cause heritable alterations in man. Direct methods for the assessment of heritable genetic defects in man do not exist. As a consequence, a wide variety of nonhuman and even non-mammalian test systems have been utilized in efforts toward the assessment of heritable damage induced in germ cells which may be of concern to man.

Certain mammalian tests are considered in and of themselves as sufficient criteria for stating that a chemical is likely to cause heritable mutations in human. These methods can assess a heritable risk and can be utilized in a manner consistent with good toxicological procedures, i.e., appropriate routes of administration, proper number for statistical purposes and dosages which approach human exposure levels. Misinterpretation of results could occur in one of these mammalian systems if the *in vivo* metabolism of the chemical in man is different than in the test system.

The tests recommended are the heritable translocation test, the *in vivo* cytogenetic test, and a specific locus test in rodents. These tests measure a variety of genetic damage. The heritable translocation test measures transmissible chromosomal aberrations. The *in vivo* cytogenetic test indicates the capacity of a particular chemical to cause chromosomal aberrations which may or may not be transmissable.

It has not yet been demonstrated that all chemicals which affect base-pair changes (gene mutations or small deletions) also cause chromosomal damage, therefore, it is essential that an assay method capable of detecting gene mutations be included. The specific locus test in mice fulfills this criterion, in that it measures mutations in specific recessive genes. Currently, there are only a few laboratories with the capacity or expertise to maintain the special strains of mice necessary for this test.

Registrants may find it useful to perform tests with other assay systems utilizing submammalian organisms and single cells for screening for a variety of genetic effects, including those which will not be detected by the whole animal tests.

ACCEPTABLE PROTOCOLS AND METHODS

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Specific Locus In vivo Test

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METHODS FOR STUDY OF NEUROTOXICITY

In the field of behavioral toxicology, techniques are being rapidly developed with experimental animals that, hopefully, will provide more sensitive and specific predictions of neurotoxic effects in man (U.S. Department of Health, Education and Welfare, 1974). Presently, gross observations for neurotoxicity are an integral part of almost every toxicity study.

Some organophosphorus compounds are known to cause a delayed locomotor ataxia in man. This neurotoxic effect has not been readily observed in rats but does occur in hens. Pesticides that are organophosphorus compounds may be studied for their ability to produce a delayed neurotoxic effect in hens (Davies et al., 1960; British Working Documents 1967).

Acceptable Protocols and Methods

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METHODS FOR STUDY OF INTERACTIONS

Pesticides are commonly prepared in formulations prior to use. The excipients of such formulations, although not usually affecting the intrinsic toxicity of the pesticide itself, may alter such characteristics as absorption. The influence of excipients may, in turn, influence the observed toxicity, resulting in an increased or decreased effect. Thus protocols for toxicity studies must evaluate the effect of the formulation as used, and care must be exercised if any extrapolation is made for an altered formulation.

Formulations of pesticides will also often contain synergists, such as piperonyl butoxide, or a mixture of one or more active pesticides. The toxic action resulting from such combinations may be independent for each compound, but it is likely that a more than additive, or less than additive, joint toxic action may be observed. The basic principles for detecting such actions, often called synergism, potentiation or antagonism, are described in the literature (Smyth et al., 1969, 1970).

The task of studying all combinations of pesticides for unwanted interactions is a physically impossible one. Fortunately, there is an understanding of the biochemical mechanism for interaction between certain combinations of pesticides that are known to produce more than additive effects. For example, it has been recommended that "studies on the inhibition of plasma and liver aliesterase should be required on all anticholinesterase pesticides" (Mrak Commission 1969; Su et al., 1971; Murphy and Cheever, 1972; Cohen and Murphy, 1974). Another important mechanism for the interaction of pesticides with other pesticides, drugs and other environmental chemicals is the alteration of the microsomal, drug-metabolizing enzymes of the liver and possibly other organs. The ability of a pesticide to induce such enzymes will often be a part of the studies on mechanisms of pesticide toxic action.

Although specific protocols must be designed for individual circumstances, it is prudent to evaluate the significance of these interactions, as man may be exposed to multiple environmental chemicals when using pesticides (Mrak Commission, 1969; Conney and Burns, 1972).

Acceptable Protocols and Methods

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METHODS FOR STUDY OF REENTRY INTERVALS

A number of sporadic episodes of acute adverse effects in field workers have been ascribed to toxic levels of pesticide residues on plant surfaces. Although no deaths have occurred, many workers have required medical care and treatment. The establishment of field worker reentry intervals appears to offer a practical approach to preventing worker exposure to hazardous residue levels.

At present it is not possible to identify, in the agricultural work place, a single or reliable indicator for establishing adequate reentry intervals. Nor is it possible to precisely enumerate all variables in the agricultural environment influencing residues and their potential availability to field workers. For these reasons, research designs, protocols and techniques are still in the developmental phase. A presentation of the present state of the art, recent experimental activity and suggested approaches may be found in the *Report of the Task Group on Occupational Exposure to Pesticides* (1974).

Establishment of reentry intervals for a specific pesticide-crop-cultural practice combination is currently conceived as a 2-step process: (1) postulating a reentry interval; and (2) testing the postulated interval in the field. Data used in step 1 must be related to the factors which are known to influence, or are suspected of influencing, the level of residues, their modification and persistence. Some of these factors are: (1) The physical and chemical properties of the applied product; (2) the method, rate and frequency of application; (3) the dermal, inhalation and oral toxicity of residues to which the worker is exposed; (4) the environmental and meteorologic conditions which affect the pesticide or its breakdown products; and (5) the route, degree and duration of worker exposure to the residues as influenced by cultural and harvesting practices for the treated crop. All available information relevant to the toxicology, degradation rate of the pesticide residues, worker exposure potential as it relates to cultural practices, and meteorological conditions are used to postulate a reentry interval.

The correlation of environmental factors as they affect cultural practices, the degree of worker exposure and the adverse response of workers to pesticide residues is not clearly understood. Therefore, at least 2 data collecting sites that differ in climatic, crop cultural and edaphic conditions must be selected because data collected in one geographical area may not be relevant to another with different environmental conditions. With proper planning, these sites may be the same sites chosen for collecting tolerance residue data.

Before applying the pesticide, the investigator should obtain the spray history of the plot to insure that pre-existing residues will not affect the experimental results. The actual application operation must be carefully supervised to insure that quantities delivered are consistent with proposed or actual label recommendations. Further, the application should be made in the season and at the frequency dictated by pest management requirements.

Meteorological data in the area containing the plot should be recorded daily between application of the pesticide and completion of the experiments. Usually there is a weather station nearby which will record daily high and low temperatures, rainfall, humidity, wind and light intensity. If the growth characteristics of the treated crop produce microenvironmental conditions which differ from those usually encountered in the area, data collection at the plot site is essential.

Chemical analysis for reentry data must include the levels of dislodgable residues for active substances applied to the crop and their major breakdown products. A dislodgable residue is one which is easily removed from the plant surface by a simple wash and is thereby distinguished from residues released from within the leaf or its surface waxes by organic solvent or homogenization. The latter are normally not available for worker exposure. Sampling must be frequent enough to define a decline curve of the residues, including any plateau which may occur with time. The final result of the analysis should be a measure of residue potential expressed in mg or μg of the pesticide and its toxic degradation products cm sq of plant surface. Samples must be collected from soil in the plot and developed in the same manner, since recent evidence indicates that dust may be a significant dermal and respiratory route of exposure for field workers. Gunther et al. (1973) present

a set of procedures for establishing dislodgable residue values on citrus. Also included in this reference are discussions of sampling procedures and a description of leaf punches used in sample collection. Although additional research may uncover other methods more predictive of the respiratory and dermal dose variables, at present these techniques appear to be most suitable.

Air sampling should be carried out while the crew is actually at work in the field. The objective is to define the respiratory dose potential at the breathing zone of the worker. Investigators should consult with experienced industrial hygienists for this phase of the experiment.

The discussion thus far is related to the data to be considered with the toxicology information in establishing a postulated reentry interval. Testing this interval is an occupational health problem requiring qualified professionals in this field. Research involving human subjects raises a host of questions; medical and ethical considerations are of particular concern. If appropriate procedures are followed, the risk to participants can be held within acceptable limits and the results can serve to reduce the hazard to all field workers exposed in the course of routine cultural operations. The recommendations made by the Task Group on Occupational Exposure to Pesticides contain guiding principles and suggestions for experimental designs in this area.

Acceptable Protocols and Methods

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SUBPART B

Hazard evaluation: Aquatic toxicology ---------- VII.B–1
Acute toxicity test methods---- VII.B–6
Chronic toxicity and partial chronic toxicity test methods. VII.B–7
Biological uptake and food chain transfer test methods---------- VII.B–29
Field testing techniques-------- VII.B–49

PART VII—HAZARD EVALUATION

SUBPART B—AQUATIC TOXICOLOGY

Terminology

Acute toxicity test refers to a test conducted to determine the concentration of toxicant in water which causes a defined effect (usually mortality, but sometimes other effects, such as reduced shell deposition in oysters) on 50 percent of the exposed population in some short time interval, typically 48 or 96 hr.

Chronic toxicity test refers to an experiment in which the test animal is cultured in the presence of toxicant from egg to egg or from one stage in the life cycle to the same stage in the next generation. Various parameters, including survival growth rate, locomotor activity, behavior changes and reproductive effects are measured to determine the effects of prolonged exposure.

Experiments extending over intermediate lengths of time are referred to as subacute toxicity tests or partial chronic tests. *Subacute toxicity* usually indicates adverse effects of a toxicant that occur after exposures of more than 1 wk but not through an entire life cycle. Examples of subacute effects, in addition to mortality, include impaired growth or locomotor activity, behavioral changes, predator avoidance, and pathological changes. A *partial chronic test* is an experiment which includes the reproductive phase but not all stages in the life cycle of one generation of the species tested.

The term *biological uptake* is used to refer to the incorporation of a pesticide into the tissue of a test species after exposure to the pesticide in water.

Food chain transfer refers to uptake through the food as distinct from uptake from water.

Because some methods have not been as well tested and accepted as others, each method included in this subpart has been characterized as routine, tentative or developmental. The characterizations are not intended to imply that a routine method is the best or only method or that a developmental method yields less valid results, but rather to indicate the degree of past usage and acceptance of a method.

1. *Routine Method.* The method has been used by innumerable toxicologists and other investigators over an extended time period with a wide variety of toxicants. Application of the method has become relatively uniform in testing laboratories, although a high degree of technical skill is necessary at least in some cases. The data output for a given compound tested in different laboratories can be expected to be similar.

2. *Tentative Method.* The method has been used by 2 or more toxicologists over a period of several years with a limited number of toxicants. There is no consensus among toxicologists on the detailed application of the method or sufficient information to show that the data output in interlaboratory tests would be consistent.

3. *Developmental Method.* The method has been proposed by 1 or a few toxicologists and used to test a few compounds by the original researcher(s). There is no consensus that the method provides appropriate data or that modifications of the method would not be appropriate. Techniques involved may not be well-known to other toxicologists, and, therefore, other toxicologists may require considerable experience with the method before they can obtain consistent results.

The tests for which protocols are presented are listed below with an indication of the category.

Toxicity Tests:	
Acute:	
Freshwater fish--------	Routine
Marine fish----------	Routine
Freshwater invertebrate	Routine
Marine invertebrate----	Routine
Chronic:	
Fathead minnows-----	Routine
Sheepshead minnow---	Developmental
Daphnia ----------	Developmental
Midge ----------	Tentative
Partial Chronic:	
Brook trout----------	Tentative
Bluegill ----------	Developmental
Accumulation Tests:	
Biological uptake--------	Tentative
Food chain transfer------	Developmental
Field Methods----------	Developmental

CONSIDERATIONS FOR MARINE TOXICITY TESTING

The general facility requirements for a toxicology test laboratory identified in the methods for freshwater systems apply also to marine systems. Whenever possible, natural sea water should be used.

Delivery of sea water to the laboratory is simple in principle but frequently difficult in fact. Subsurface water is typically pumped into the laboratory through 2–4 in. plastic pipes (PVC with PVC or nylon connectors) to prevent introduction of toxic metals. The pump should have a stainless steel impeller. No other metal should be in contact with the sea water. The volume of flow should exceed the volume required for all tests by a considerable margin.

Because fouling organisms, such as barnacles, sea squirts, oysters, etc., will enter the lines as larvae and settle in the pipes and reduce flow and increase turbidity, a dual system of pump and pipes is usually installed. One line may then be turned off, drained, filled with fresh water, or simply let stand for a period, and back flushed, while the other line supplies the laboratory. Lines should be rotated on a regular basis to safeguard against fouling and to exercise the pump. Details of systems found practical in various situations are described by Clark and Clark (1964), but improved systems continue to be designed (DeBen 1970; White et al. 1973). Various marine laboratories around the country can advise on system design.

Natural sea water should, *at a minimum*, permit the maintenance of healthy test organisms for the period required for laboratory acclimation and testing. Since oyster larvae or copepods are more sensitive to many toxicants than other species, a more realistic criterion for acceptable sea water is that these species should survive and/or develop normally for 1 wk. Several other criteria should also be met if possible. The salinity of the water should be greater than 15‰, with tidal variations less than 5‰. Turbidity should be minimal to simplify water delivery. Other criteria for diluent water as set forth in Stephan et al. (1975) should also be met.

In cases where salinities are less than 15‰ or diurnal variations are great (greater than 5‰), ancillary equipment should be installed to maintain the salinity of the incoming water at a desired level. This can be accomplished by controlled addition of a brine prepared with an artificial sea salt mixture (Wood 1965; DeBen 1970). In situations where high turbidity is encountered, filtration may be desirable, using either a rapid sand system (White et al., 1973) or industrial filter cartridges. The water may be sterilized by passage over ultraviolet light (e.g., Shelbourne 1962). Commercial ultraviolet sterilizers are also available.

Instrumentation is necessary to determine salinity on a routine basis. Of the methods available, titration of chloride with silver nitrate (Strickland and Parsons, 1968), refractometric methods or the conductivity method are preferable because of their accuracy. The latter method has the greatest accuracy, requires the least amount of time and can be used as an on-line monitor. Conductivity salinometers are commercially available. A supply of Standard Sea Water is necessary for calibration purposes (available from the Supply Department, Marine Biological Laboratory, Woods Hole).

Acute tests in static systems can be conducted with artificial sea water, since only small volumes of water are required. Several artificial sea salt preparations are available commercially. Instant Ocean® (Aquarium Systems, Inc.) has been shown by many investigators to be suitable for the culture of many, though not all, marine invertebrates and fishes. However, variability in the salt mixture may arise as a result of changes in the formulation, and all preparations should be tested for toxicity before use in toxicity tests.

Commercial artificial sea water preparations have been found inadequate for studies involving some marine molluscs and especially their larvae. Several suitable formulations from technical grade chemicals have been reported in the literature. An appropriate formula is included in Stephan et al. (1975).

The report by Stephan et al. (1975) indicates the desirability of testing marine organisms at salinities of 5, 15 and 35‰ to determine the effect of salinity on acute toxicity. It should be noted that these salinities are outside the tolerance range of some species. The range of salinity tested should be

within the tolerance range of the test species, and the test animals should be carefully acclimated several days prior to the test.

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Strickland, J. D. H. and T. R. Parsons. 1968. A practical handbook of seawater analysis. Fish. Res. Board Can. Bull. No. 167. 311 pp.

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ACUTE TOXICITY TEST METHODS

Bahner, L. H., C. D. Craft and D. R. Nimmo. 1975. A saltwater flow-through bioassay method with controlled temperature and salinity. *Progressive Fish Cult.* (In press).

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Three methods are included: The bivalve embryo test; the shell deposition test; and the long-term growth test. This paper is currently undergoing final revision and will be provided when available. Available from Dr. P. A. Butler, Gulf Breeze Environmental Research Laboratory, Sabine Island, Gulf Breeze, Florida 32561.

Stephan, C. E., chairman, Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. U.S. E.P.A., Ecol. Res. Ser. (In press).

A number of "recommended" species are listed in this publication, most of which would be acceptable to EPA. An exception is the goldfish, *Carassius auratus*. Bluegill and rainbow trout remain the preferred species for acute toxicity tests.

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CHRONIC TOXICITY AND PARTIAL CHRONIC TOXICITY TEST METHODS

Biesinger, K. E. and G. M. Christensen. 1972. Effects of various metals on survival, growth, reproduction and metabolism of *Daphnia magna*. *J. Fish. Res. Board Can.* 29:1691–1700.

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Biesinger, K. E. 1974a. Tentative procedure for *Daphnia magna* chronic tests in a standing system. EPA National Water Quality Lab., Duluth, Minnesota. (Exhibit 1).

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———1974c. Culturing methods for *Daphnia* and certain other cladocerans. EPA National Water Quality Lab., Duluth, Minnesota. (Exhibit 3).

Derr, S. K. 1974. Tentative chronic bioassay procedure for the midge *Chironomus tentans*. (Exhibit 4).

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Schimmel, S. C. and D. J. Hansen. 1974. Sheepshead minnow (*Cyprinodon variegatus*): An estuarine fish suitable for chronic (entire life-cycle) bioassays. *Proc. S.E. Assoc. Game Fish Comm.* (In press).

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EXHIBIT 1

TENTATIVE PROCEDURE FOR DAPHNIA MAGNA CHRONIC TESTS IN A STANDING SYSTEM

By Kenneth E. Biesinger. EPA National Water Quality Laboratory, Duluth, Minnesota

A. *Physical system*

1. *Test containers.* Two-hundred and fifty ml beakers containing 200 ml of test water are recommended.

2. *Toxicants.* Toxicant mixing is best accomplished in one or two liter flasks which can then be poured into test containers. The toxicant must be renewed each week; this is best accomplished by setting up clean beakers with the food and toxicant added, and then transferring adult daphnids. Analytical testing of toxicant concentrations must be made at the beginning and at the end of each week; if the compound is volatile then intermediate analysis are needed.

3. *Duplication.* For each test four beakers each containing five daphnids must be run for each concentration tested as well as for the control (i.e., the test must be run in quadruplicate).

4. *Replication.* Three separate tests must be for each toxicant.

5. *Aeration.* Aeration is not recommended.

6. *Cleaning.* All glassware must be thoroughly washed and rinsed with distilled water. Test containers and flasks must have an additional three rinses with the water to be tested just before a test is to be initiated. For testing of metals an intermediate half hour soaking of glassware in 20% nitric acid is needed. For testing of organic compounds an intermediate thorough rinsing of glassware by acetone is needed.

7. *Solvents.* If solvents are used with organic toxicants the same amount of solvent must be added for each concentration of the toxicant to be tested. In addition to a water control a solvent control must also be tested.

8. *Light and photoperiod.* Fluorescent bulbs using a combination of Durotest (Optima) and wide spectrum Grow-lux bulbs with a 16 hour photoperiod (automatically controlled) are recommended.

9. *Temperature.* A temperature of 18±1° C is recommended.

10. *Toxic materials.* Use of dechlorinated tap water is not recommended; a concentration of less than 1 μg/1 chlorine will result in death to daphnids. Rubber and many other commonly used laboratory apparatus are toxic to daphnids; the toxicity of any questionable material may be tested for three weeks and, if there is something toxic, it will be evident by noting a reduction in young production if not mortality. Plastics are not suitable for testing of organic compounds. Silicone rubber cement is nontoxic with sufficient leaching.

B. *Biological system.*

11. *Acclimation.* Laboratory raised animals may be tested at any time. *Daphnia* collected in the field should be reared through two generations before commencing chronic studies.

12. *Food and feeding.* A suspension of food (1 ml/1) should be fed once weekly when the toxicant is renewed. We recommend adding a suspension of food containing Cerophyll and trout fry granules. The food suspension is prepared by weighing out 0.5 grams of Cerophyll and 10 grams of enriched trout fry granules. These are added to 250 ml of lake, well, or river water and mixed vigorously in a blender for five minutes. The suspension is then strained through stainless steel screen (105 mesh with 0.165 inch openings); and an additional 50 ml of water is used to rinse the blender and added to the above suspension. The food thus prepared is stored in a refrigerator and thoroughly mixed each time an aliquot is withdrawn.

13. *Testing methods.* It is recommended that young daphnids, 12±12 hours old (if reared at 18°C) be placed in test chambers and subjected to test conditions for three weeks. Daphnids should be transferred with an eye dropper into clean containers each week. Survival of the initial animals added should be noted after one week, two weeks, and three weeks. Reproduction should be noted by counting the young produced each week; the young should be counted and discarded each time the initial animals are transferred and at the end of each experiment. It is recommended that five daphnids be placed in 200 ml of water in 250 ml beakers. Four beakers should be used with a total of 20 animals for each experimental condition for each test. The beakers must be randomly distributed on a shelf and re-randomized each week. The beakers must be covered with a pane of glass and placed in a tray of water to minimize evaporation.

14. *Concentrations.* The number of concentrations to be tested should be from four to eight depending on the toxicant and the effects to the initial animals as well as to the effects on reproduction. Initial concentrations tested should be designed to bracket published results (i.e., above and below); however, as daphnids have not been tested on a chronic basis to many toxicants, experiments often have to be repeated before arriving at "no effect" levels. After delineating the concentrations to be tested, three successful tests should be run for each toxicant in which sixty animals would be tested for

each concentration. Adult survival in the control water must be 70% or more after three weeks for the test to be valid.

15. *Statistical evaluations.* Statistical evaluations of results for adults may be made using the method of Litchfield and Wilcoxon (1949) for establishing approximations of the 95% confidence limits for survival. Semilogarithmic probability paper is used for analyzing results. The terminology recommended by Sprague (1969) is used for survival (lethal concentration=*LC*) and represents three or more partial effect concentrations.

Statistical evaluations of results for young may be made using an Analysis of Variance on the young produced during three weeks of exposure by combining three tests and determining the sources of variation due to: Experiments, treatments, and experiments X treatments. Dunnett's Test ($P=.05$) is recommended to find the concentrations significantly different from the control.

References

Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* 96: 99–113.

Sprague, J. B. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. *Water Res.* 3:793–821.

EXHIBIT 2

TENTATIVE PROCEDURE FOR DAPHNIA MAGNA CHRONIC TESTS IN A FLOWING SYSTEM BY KENNETH E. BIESINGER. EPA NATIONAL WATER QUALITY LABORATORY, DULUTH, MINNESOTA

A. *Physical system.*

1. *Diluter.* Proportional diluters (Mount and Brungs, 1967) (or other suitable delivery devices) should be employed for testing to obtain the most toxic conditions. A series of two diluters should be employed: the first for adding food and the second for adding toxicants. If solvents are added then modifications of the diluter should be made to enable adding the same amount of solvent for each concentration; this is most easily accomplished by having separate toxicant injectors for each concentration to be tested.

2. *Toxicant mixing.* A container to promote mixing of toxicant with water should be used between the diluter and the tanks for each concentration. Separate delivery tubes should run from this container to each quadruplicate tank. The same amount of water and toxicant must be added to each quadruplicate tank; the same volume must be added to all tanks.

3. *Quadruplicates.* Use true quadruplicates for each tank concentration (there should be no tank water connections between any of the four tanks). The location of each test tank should be determined by stratified random assignment.

4. Each tank, preferably all glass (we use battery jars), should contain at least two liters of water. Drainage is most easily accomplished by cutting a two inch notch at the top of the jars and attaching stainless steel screen (105 mesh with 0.165 inch openings) by silicone rubber cement over the openings.

5. *Flow rate.* Flow rates for each tank should be 2–4 tank volumes/24 hrs. If a diluter is adding more than 50 ml/cycle then a purge chamber is recommended (i.e. an intermediate tank having a siphon going into each test tank).

6. *Aeration.* Aeration is not recommended. Total dissolved oxygen levels should never be allowed to drop below 80% saturation.

7. *Cleaning.* The diluters and test tanks must be thoroughly cleaned once each week. The test tanks are most easily cleaned by setting up new tanks and transferring daphnids with an eye dropper.

8. *Light and photoperiod.* Fluorescent bulbs using a combination of Durotest (Optima) and wide spectrum Grow-lux bulbs with a 16 hour photoperiod (automatically controlled) are recommended.

9. *Temperature.* A temperature of $18\pm1°C$ is recommended.

10. *Toxic materials.* Testing in dechlorinated tap water is not recommended; a concentration of $<1\mu g/l$ chlorine will result in death to daphnids. Rubber and many other commonly used laboratory apparatus are toxic to daphnids; the toxicity of any questionable material may be tested in standing systems for three weeks and, if there is something toxic, it will be evident by noting a reduction in young production if not mortality. Plastics are not suitable for testing of organic compounds. Polyvinyl chloride tubing and silicone rubber cement are nontoxic with sufficient leaching and therefore, these materials along with glass are recommended for all testing.

B. *Biological system.*

11. *Acclimation.* Laboratory raised animals may be tested at any time. *Daphnia* collected in the field should be reared through two generations before commencing chronic studies.

12. *Food and feeding.* A suspension of food should be fed continuously. We recommend adding a suspension of food containing powdered dried grass or alfalfa leaves and trout fry granules. The amount to add will depend on the organic enrichment of the water being tested. The food suspension is prepared by weighing out 0.5 grams of powdered dried grass and 10 grams of enriched trout fry granules. These are added to 250 ml of lake or river water and mixed vigorously in a blender for five minutes. The suspension is then strained through stainless steel screen (105 mesh with 0.165 inch openings); and an additional 50 ml of water is used to rinse the blender and added to the above suspension. The food thus prepared is stored in a refrigerator and thoroughly mixed each time an aliquot is withdrawn. A mariotte bottle with a mechanical stirrer is used to deliver food to the diluter; the food suspension is renewed every three days.

13. *Testing methods.* It is recommended that young daphnids, 12 ± 12 hours old be placed in test chambers and subjected to test conditions for three weeks. Daphnids should be transferred with an eye dropper into cleaner containers each week. Survival of the initial animals added should be noted after one week, two weeks, and three weeks. Reproduction should be noted by counting the young produced each week; the young should be counted and discarded each time the initial animals are transferred and at the end of each experiment. It is recommended that five daphnids be placed in 2 liters of water in battery jars. Four battery jars should be used with a total of 20 animals for each experimental condition for each test. The battery jars must be randomly distributed on a shelf.

14. *Concentrations.* The number of concentrations to be tested should be from four to eight depending on the toxicant and the effects to the initial animals as well as to the effects on reproduction. Initial concentrations tested should be designed to bracket published results (i.e., above and below); however, as daphnids have not been tested on a chronic basis to many toxicants, experiments often have to be repeated before arriving at "no effect" levels. After delineating the concentrations to be tested, three successful tests should be run for each toxicant in which sixty animals would be tested for each concentration. Adult survival in the control water must be 70% or more after three weeks for a test to be valid.

15. *Statistical evaluations.* Statistical evaluations of results may be made using an Analysis of Variance on the young produced during three weeks of exposure by combining three tests and determining the sources of variation due to: Experiments, treatments and experiments X treatments. Dunnett's Test ($P=.05$) is recommended to find the concentrations significantly lower than the control. For adult survival either the method of Litchfield and Wilcoxon (1949) or that given in American Public Health Ass'n "Standard Methods" may be used.

References

American Public Health Association, Inc. 1965. Standard methods for the examination of water and wastewater, 12th Ed. Am. Public Health Assoc., New York. 769 pp.

Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* 96:99–113.

Mount, D. I. and W. D. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. *Water Res.* 1:21–29.

EXHIBIT 3

CULTURING METHODS FOR DAPHNIA AND CERTAIN OTHER CLADOCERANS BY KENNETH E. BIESINGER. EPA NATIONAL WATER QUALITY LABORATORY, DULUTH, MINNESOTA

Cladocerans are cultured in lake water[1] (strained through stainless steel screen having 105 mesh with 0.165 openings), in a constant temperature room at $18.5\pm1°C$ using a 16 hr automatically controlled photoperiod. Duro-Test Optima and Gro-Lux fluorescent bulbs are used to provide a light intensity of ~115 ft c at the air-water interface. Approximately 5–10 daphnids of mixed age (young and adults) are transferred weekly (using a widemouth medicine dropper) into each liter of water which has been allowed to equilibrate to room temperature and pressure for at least 24 hrs. The animals are fed one ml food[2] per liter of water when transferred and again after three or four days. *For Daphnia magna, D. pulicaria* (pulex) and other large cladocerans, glass jars holding ~8 liters of water are used; for smaller cladocerans, glass jars holding from 1–2 liters of water are used. For each species currently being used for tests, from 10–20 glass jars are maintained from which some animals (above) are transferred weekly into clean containers. Some week old cultures are kept an additional week as spare brood stocks and then discarded, although animals from these are not used for tests. By keeping cladocerans in the growth phase at all times overcrowding, male production, ephippia (resting eggs) formation, and "crashes" are prevented. Transferring the animals weekly into clean solutions also helps control excessive bacteria and algae growths.

Twenty-four hours before setting up a test, from 20–40 adult cladocerans with large eggs are transferred from culture jars into other jars containing food and water (1–2 liters). The young produced during the following 24 hr time period are used for starting all tests by transferring them into control and test solutions in intermediate containers (50 ml beakers) and then transferring them again into the same control and test solutions in containers to be used for tests.

For routine tests either *D. magna* or *D. pulicaria* (often misidentified as *D. pulex*) are recommended. In addition to chronic tests

(attached), two to four day acute tests are recommended. Acute tests may be evaluated using the Litchfield and Wilcoxon (1949) method for 50% lethal concentrations (LC-50's), or "Standard Methods" for median tolerance limits (TLM's).

Footnotes

[1] Well, pond, or river water may also be used. Dechlorinated tap water is not recommended; a concentration of <1 μg/l chlorine may result in death to daphnids.

[2] The food suspension is prepared by weighing out 0.5 grams of Cerophyll and 10 grams of enriched trout fry (Glencoe Mills) granules. These are added to 250 ml of lake, well, or river water and mixed vigorously in a blender for five minutes. The suspension is then strained through stainless steel screen (105 mesh with 0.165 inch openings) forcing the smaller particles through with a spatula; and an additional 50 ml of water is used to rinse the blender and added to the above suspension. The food thus prepared is stored in a refrigerator for up to one week and thoroughly mixed each time an aliquot is withdrawn.

References

American Public Health Association, Inc. 1965. Standard methods for the examination of water and wastewater, 12th Ed. Am. Public Health Assoc., New York. 769 pp.

Litchfield, J. T., Jr. and F. Wilcoxon, 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* 96: 99–113.

EXHIBIT 4

TENTATIVE CHRONIC BIOASSAY PROCEDURE FOR THE MIDGE "CHIRONOMUS TENTANS" BY S. KROGH DERR. BIONOMICS, EG AND G, INC., WAREHAM, MASSACHUSETTS

MARCH, 1974.

A. *Physical Exposure Systems.*

1. Use a proportional diluter for the chronic exposure system as described by Mount and Brungs (1967) and modified to deliver 500 ml of experimental water per dilution cycle. A container is necessary to promote mixing of the toxicant solutions before delivery to the test aquaria. Separate delivery tubes should lead from the toxicant mixing cells to the respective experimental chambers.

2. *Experimental Chambers.* Use true duplicate aquaria for each toxicant concentration. Placement of each aquarium should be determined by a stratified random assignment. Experimental aquaria should be constructed with all glass and be of such dimension to hold 3 liters of water. Adequate screening (aluminum) should be placed over each aquarium to retain emergent adults. The flow rate to each chamber should be maintained at a rate equal to 3 to 5 tank volumes every 24 hours.

3. *Temperature.* Diluent toxicant water in the aquaria should be maintained at 22± 1°C and never be allowed to exceed 25°C.

4. Dissolved oxygen should be above 40–50% saturation.

5. *Photoperiod.* Maintain exposure day lengths at a 12-hour light-dark cycle. Use a combination of Gro-Lux (wide-spectrum) and Durotest (optima F8) lights providing an intensity at the water surface of 70–140 foot candles illumination.

B. *Biological Systems.*

1. *Holding.* Maintain all experimental animals in a 40–60 l all-glass aquarium. The holding chamber should be adequately supplied with a defined substrate and aerated.

2. *Food–Defined Substrate.* A substrate of homogenized paper hand towel (Nibroc Co.) and high protein poultry or trout ration is required. The substrate is prepared by homogenizing 50 g of hand towel and 5 g of the selected food with enough water to cover the mixture in a Waring Blendor. The substrate is then placed into each experimental aquarium at a depth of ½ inch.

3. *Analytical Chemistry.* Concentration of toxicant will be measured at least weekly in each experimental unit, by appropriate analytical method to determine actual exposure levels.

4. *Initiation of Chronic Test:* (First Generation). Place one hundred eggs from 1 egg mass into an egg cup suspended in each experimental aquarium (12 aquaria total). After a 3-day period record the percent hatchability and pipette the first instar larvae onto the defined substrate.[1] Allow the larvae to develop, pupate and emerge in the same time period as the controls; any delayed or retarded developmental time in any of the experimental aquaria would be ascribed to treatment. Collect egg masses from those treatments that realized egg deposition in the same time period as the controls and initiation of the second generation in the same manner as the first. Additional food-enriched substrate should be added to test aquaria after first generation emergence occurs.

EXPERIMENTAL INDICES INCLUDE

FIRST GENERATION

1. Percent hatch
2. Number of pupae
3. Number of adults
4. Reproduction and egg deposition
5. Developmental time of larvae when compared to controls

SECOND GENERATION

6. Percent hatch
7. Number of pupae
8. Number of emergent adults
9. Developmental time of larvae when compared to controls

SELECTED REFERENCES

Derr, S. K. and M. S. Zabik. 1972. Biologically active compounds in the aquatic environment: The uptake and distribution of DDE by *Chironomus tentans*, (Diptera: Chironomidae) Trans. Amer. Fish. Soc. 101:323–329.

Mount, D. I. and W. D. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. Water Res. 1:21–29.

Sadler, W. O. 1935. Biology of the midge, *Chironomus tentans* Fabricius, and methods for its propagation. Memoir 173, Cornell University, New York.

EXHIBIT 5

ENTIRE LIFE-CYCLE BIOASSAY USING SHEEPSHEAD MINNOWS (CYPRINODON VARIEGATUS)* BY DAVID J. HANSEN AND STEVEN C. SCHIMMEL. EPA, GULF BREEZE ENVIRONMENTAL RESEARCH LABORATORY, GULF BREEZE, FLORIDA

A. *Purpose.* The purpose of this procedure is to provide a method of determining the effect of a material on survival of sheepshead minnow embryos and fry, their growth to adulthood, and reproductive success. Reproductive success is measured by monitoring the ability of pairs of fish to spawn naturally, their fecundity, fertilization success and survival of embryos and fry.

B. *Physical Systems*—1. *Water source.* a. The source of test water should be natural seawater with salinity ≧ 15 °/₀₀. It may be acceptable to add artificial sea salts to achieve a salinity of ≧ 15 °/₀₀ using freshwater or natural seawater with a salinity of < 15 °/₀₀.

b. Seawater must be filtered to remove particles 15μ and larger, but filtration should not affect the chemical composition of the natural seawater. Filtration must remove planktonic larvae which could prey upon eggs, fry and juvenile fish in the chronic bioassay.

c. Any water source proposed must be analyzed for possible pollutants (e.g., pesticides, PCBs and heavy metals). Special determinations should be made for those toxicants being investigated.

2. *Dosing apparatus.* A number of apparatus would be acceptable for this bioassay including those of Mount and Brungs, 1967; Hansen et al., 1971; Hansen et al., 1974 or Schimmel et al., 1975.

3. *Toxicant mixing.* A mixing chamber is necessary to assure adequate mixing of the test material. Aeration should not be used for mixing.

4. *Duplicates.* True duplicates should be used for each concentration in all tests (no water connection between aquaria). Aquaria location should be by random selection.

5. *Aquaria.* Each duplicate glass aquarium should be large enough to allow for growth and reproduction. Aquaria measuring 30x90x 30 cm high and having a water depth of 15 cm have been used successfully.

6. *Embryo and fry chambers.* a. Embryo and fry chambers should be constructed to allow for adequate exchange of water and to insure that the proper quantity of material is entering the chambers. Embryo chambers (incubation cups) can be constructed from 113 g., 5 cm, OD round glass or beakers with the bottoms cut off. The bottoms are replaced with 40 mesh stainless steel or nylon screening. Chambers are hung on an oscillating rocker arm apparatus that is driven by a 1–5 rpm electric motor (Mount, 1968). These chambers must be brushed daily to prevent clogging. Chambers may also be constructed from Petri dishes to which nylon or stainless steel screen is glued (Hansen *et al.*, 1974).

b. Embryo and fry chambers should be designed so that the water can be drained down to 2.5 cm (1 inch) in order to facilitate growth measurements of fry.

c. Embryo and fry chambers, volume ≧400 ml, may be supplied test water by (1) separate delivery tubes from the mixing chamber, (2) splitting the flow from the aquaria or (3) egg cups on a "rocker" arm.

7. *Flow rate.* a. Flow rates to each duplicate aquaria should be great enough to (1) provide 90% replacement in 8–12 hours (Sprague, 1969); (2) maintain dissolved oxygen, 60% saturation; and (3) maintain the toxicant concentration.

b. The test system should be equipped with an alarm system to insure continuation of water and toxicant flow.

8. *Photoperiod.* A 16-hour light/8-hour dark cycle should be maintained throughout the test. It may be desirable to control lights by a timing switch (Drummond and Dawson, 1970).

9. *Temperature.* Test temperature should be maintained at 30°C (±1 C). Temperatures can be maintained by (1) preheating the diluent water to the prescribed temperature, and/or (2) placing the test aquaria in a temperature controlled water bath.

10. *Cleaning.* All aquaria should be cleaned whenever organic material builds up. Aquaria should be brushed down and siphoned to remove accumulated material. To reduce stress, fish can be left in aquaria. However, care

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[1] After pipetting the first instar larvae onto the substrate, take exceptional precautionary measures not to disturb the larvae after they have constructed their respective tubes.

*This procedure has been used at the Gulf Breeze Environmental Research Laboratory. This is a preliminary draft. It has not been formally reviewed by this laboratory and is being circulated for information only.

should be exercised in cleaning to prevent loss or damage to the fry, juveniles or adults. Embryo and fry chambers may need to be cleaned frequently or replaced when screens clog or organic material collects. Special care is required to prevent injury to fry.

11. *Spawning chambers.* Chambers should be constructed of either glass or #316 stainless steel. Chambers should be large enough to allow the male to establish a territory and the female to escape attacks by the male. Chambers measuring 13x30x15 cm high, with a large mesh screen (1 cm^2) attached 1 cm above the bottom to enable eggs to pass through, have been used successfully. The mesh screen on the bottom reduces predation by the parents on the eggs.

12. *Disturbance.* All test chambers containing fish should be shielded from excessive outside disturbances. Tanks should be shielded from all outside light sources that would interfere with the photoperiod.

13. *Concentrations.* a. A minimum of 5 concentrations of toxicant and a control, all duplicated, should be utilized in all acute and chronic tests.

b. Concentrations selected for chronic toxicity experiments should be based on results of acute flow-through bioassays. Concentrations should be selected so that at least one will adversely affect some life stage of the sheepshead minnow and one will not affect any stage.

c. Chemical analyses should be made of the toxicant to be tested, of the water during this test and of the fish during and at the conclusion of the test. At a minimum, the concentration of toxicant in the test water of each test concentration should be analyzed weekly. Each life stage (eggs, fry, juvenile and adult fish) from each toxicant concentration should be analyzed during the test. Whole adult fish, flesh, eggs or ovaries should be analyzed.

14. *Acute tests.* Acute flow-through bioassays should be conducted prior to initiation of any chronic test (Sprague, 1969). It is desirable for these tests to be on at least two different age classes (e.g., fry, juveniles or adults).

C. *Biological Systems*—1. *Source of adult fish.* Adult fish should be obtained from the same source, either from wild population or suitable culture laboratories. They should be held in flowing 30° C sea water of ≥15 °/₀₀ salinity for a minimum of two weeks before obtaining eggs.

2. *Obtaining eggs from adult fish.* To obtain a sufficient number of eggs to begin a chronic exposure, two methods may be employed. (1) Natural spawning from laboratory stocks. (2) Artificial inducement, where adult female egg production is stimulated by injection of human gonadotrophic hormone and fertilized with sperm excised from males. (Schimmel et al., 1974). The former may be preferable.

3. *Beginning the test.* Two groups of fifty fertilized eggs are placed in two or more separate embryo and fry chambers in each duplicate aquarium. Survival of embryos and fry (which constitute the parental stock, F_1) are to be checked and recorded daily. After 4 weeks fish are reduced to 25 per duplicate aquarium. This should provide enough fish so that at least five pairs of adults will be available in each replicate aquarium for spawning.

4. *Food.* a. Fry should be fed live brine shrimp nauplii (*Artemia salina*) two or more times daily for about 2 weeks. (Do not use frozen nauplii.) Automatic feeders are recommended (Schimmel and Hansen, 1975). After 2 weeks, supplement with dry trout pellets (e.g. Oregon Moist) or dry mollie flakes (e.g. Biorell) for 2 additional weeks.

b. Juveniles and adults can be fed twice daily dry food supplemented with frozen adult brine shrimp. Each batch of food should be checked for pesticides (DDT, dieldrin, endrin, etc.). In addition, chemical analysis should also include chemicals in the material to be tested.

5. *Disease.* Disease outbreaks should be handled according to their nature with each aquarium being treated similarly, even though disease is not evident in all aquaria. All treatments should be kept to the minimum and recorded as to type, amount and frequency.

6. *Measuring fish.* A sample of fish of the F_1 generation should be measured at one day post hatch, 4 weeks, 8 weeks, and at adult termination. In addition, fish are to be weighed at week 4 and termination. Larval (F_2) fish are to be measured at 1 day post-hatch and weighed and measured at week 4 (termination). Suggested techniques for measuring fish include the photographic method outlined by McKim and Benoit (1971), or placing fish in a beaker or petri dishes containing .63 cm (¼ inch) of water on a photocopier.

7. *Thinning.* At 28 days post-hatch, the F_1 larvae should be randomly reduced to 25 fish per duplicate aquaria.

8. *Spawning chambers.* As mature adults (F_1) begin courtship (indicated by sexual dimorphism, territoriality, aggressive behavior and courtship by the male), separate pairs should be placed in individual spawning chambers in the aquaria. Pairs should be left in the chambers until a sufficient numbr of eggs have been collected to insure statistical comparisons of fecundity, fertility, and survival of embryos and fry can be made.

9. *Removing eggs.* a. All eggs should be removed at a fixed time each day so the fish are not overly disturbed and disruption of spawning activity will not occur.

b. Daily records of spawnings, egg numbers, and egg fertility must be kept. All eggs must be removed daily, examined for fertility, and then used for survival studies or residue analyses or discarded. Each pair should be observed daily for a minimum of two weeks.

10. *Egg incubation.* a. A series of groups of 50 fertile eggs should be collected and incubated from adults in each aquarium. Preferably, they should be obtained from one daily spawn from each of the five spawning pairs. If necessary, because of small spawns, eggs may be collected over an extended period in order to obtain 50 eggs.

b. If no spawning occurs in the higher concentration, eggs should be transferred from control spawns and incubated in the higher concentration to gain additional information.

c. Groups of fifty eggs are placed in two or more egg cups. Survival of embryos, time required to hatch, hatching success, and survival of fry for 4 weeks will be determined and recorded.

d. Additional groups of 50 eggs from fish from contaminated aquaria should be placed in control aquaria to determine if they contain chemicals toxic to embryos or fry.

e. Remaining eggs should be analyzed for toxicant residue.

11. *F_2 embryos and fry.* Survival of embryos and fry will be recorded daily for 4 weeks. Fry are to be measured on 1 day post hatch and weighed and measured on week 4 (termination). Daily records should be kept on mortalities, development of abnormalities and behavioral changes.

12. *Termination of adults.* a. With many chronic procedures utilizing other fishes, termination of the test is considered the time when no spawning activity has occurred over a 2 week interval. For the sheepshead minnow, however, termination should occur after 2 or more weeks of spawning. Enough spawns have to occur to statistically predict the effect of the toxicant that is tested. It is our experience that most fish will spawn readily and almost daily unless sick, affected by a pollutant or immature.

b. Adult fish should be weighed, measured, sexed and retained for residue analysis.

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TENTATIVE BIOLOGICAL UPTAKE AND FOOD CHAIN TRANSFER TEST METHODS

Johnson, B. T., and R. A. Schoettger. 1974. A biological model for estimating the uptake, transfer, and degradation of xenobiotics in an aquatic food chain. Fish-Pesticide Research Laboratory, Columbia, Mo. (Exhibit 1).

This method deals with food chain transferral of pesticides.

Macek, K. J., M. E. Barrows and B. H. Sleight, III. 1974. Bioconcentration of ^{14}C-pesticide by bluegill sunfish during continuous aqueous exposure.

Only the introduction and methodology sections of this paper are currently available. The method is currently being revised in a more complete form and will be provided when available. Copies of this methodology in present form may be obtained from Dr. Kenneth J. Macek, Bionomics, EG and G Environmental Consultants, Wareham, Massachusetts.

Exhibit 1

A BIOLOGICAL MODEL FOR ESTIMATING THE UPTAKE, TRANSFER, AND DEGRADATION OF XENOBIOTICS IN AN AQUATIC FOOD CHAIN

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Introduction

The protocol described here is designed to investigate the uptake, transfer, and degradation of xenobiotics in a simple food chain representing three aquatic trophic levels. The model food chain is, in essence, a simple means to estimate under controlled conditions, the movement of an organic synthetic chemical (i.e. a "foreign" molecule or xenobiotic) by certain representative trophic levels of a natural aquatic ecosystem. The model conforms to selected criteria; it is inexpensive, simple to maintain, reproducible, ecologically relevant and produces clearly definable data. Each trophic level must be evaluated if meaningful information concerning xenobiotic uptake is to be derived from the model at the vertebrate trophic level. We wish to know how much of it is taken up. How much is retained? What is the quantity and quality of the residue? What is the uptake rate? How much can be moved via natural food and direct water uptake? What are the maximums of all these?

This model is compartmentalized or modular as compared with the microcosm system of Metcalf and others (Metcalf *et al.*, 1971; Reinert 1972; Isensee *et al.*, 1973; Matsumura and Beneset 1973). The warm water (>16C) food chain consists of three trophic levels with fish representing the top level: (1) microorganisms (yeast, protozoa, bacteria or algae) ➤ (2) filter feeder (daphnid) ➤ (3) fish (bluegill). Potentially, information may be drawn from five points within the model system. For example, in the warm water food chain, data may be gathered on uptake along several routes from water to microorganisms, microorganisms to daphnids, water to daphnids, daphnids to fish, and water to fish. Also the simultaneous use of different water and food combinations produces a number of possible permutations. One may use part or all of the food chain to evaluate potential uptake of the xenobiotic. The three segments are never tested together in the same container because of the difficulty in controlling the feeding rates (food chain transfer) between trophic levels.

Both quantitative and qualitative information are obtained from the food chain. One can estimate from the model the rate of uptake of a chemical by direct uptake from water (respiratory structures and body surface) or by uptake from residues in natural food, and also determine the chemical changes in the residue caused by abiotic or biotic systems.

Concentration Selection and Relevancy of Exposure

The concentration(s) of the xenobiotic under investigation should be relevant to potential use of the information for registration or environmental impact statement reviews. Select the concentrations for food chain studies on the basis of acute toxicity, recommended use rates, or information on probable concentrations likely to occur in aquatic ecosystems. Acute toxicity data, or LC50 (lethal concentration) values probably represent the best information at present on which to base the selection of concentrations. LC50 data are usually readily available and reproducible within certain confidence limits. Select the LC50 that represents the least tolerant member of the food chain model. Concentrations of the xenobiotic used in the uptake studies should not exceed the LC50, even though an uptake plateau or equilibrium level is reached in less than the 48 or 96-hr duration of standard acute toxicity tests. Use a concentration between 1/10 and 1/1000 of the LC50, depending on the slope of the toxicity curve. For example, if the daphnids are the most sensitive species to the xenobiotic and have a 48-hr EC50 of 1.0 μg/1, we suggest use of a test concentration between 0.1 μg and 0.001 μg/1 for the model. However, other sublethal concentrations are preferable if they can be estimated from anticipiated use rate, or from a concentration projected or measured in aquatic ecosystems.

Chemical Analysis

^{14}C-labeled compounds

Chemical analyses used in measuring uptake and degradation require sensitivity sufficient to detect and quantify nanogram amounts. Therefore, we recommend radiolabeled compounds and radiometric assay consisting of liquid scintillation spectrometry and autoradiography of thin-layer chromatograms. The radioactive material, preferably ^{14}C-labeled, should occupy the most stable portion of the molecule. Efficiency of the radiometric system is based on comparison with a spiked control. Quench curves should be plotted with different microbes, daphnids, and fish.

Xenobiotic separation and identification

Extraction. Take 100 mg of either microorganisms or daphnids, or 500–1500 mg of fish and homogenize in a tissue grinder with 5–10 ml of a selected extraction solvent (Hesselberg and Johnson, 1972). Pour the homogenate into a small porcelain evaporation dish and blend with anhydrous Na_2SO_4 (I: 4 w/w) to form a free flowing dry mixture. Pour the mixture into a 20 x 400 mm chromatography column plugged with a small piece of glass wool at the bottom of the column and lightly pack the column with the Na_2SO_4 mixture. Wash the grinding tube with the extracting solvent and add a sufficient amount of solvent to the chromatography column reservoir to insure maximum extraction efficiency. Concentrate the column effluent by evaporation to ca 1.0 ml in a porcelain evaporation dish with heat and a gentle stream of nitrogen under a fume hood. Pour the concentrated solution into a 5 ml centrifuge tube and carefully evaporate to 0.1 ml.

Thin-layer Chromatography. Spot the total sample on an appropriate thin-layer plate with a 10 μl Hamilton syringe and develop the plate in an appropriate solvent system. The extraction solvent, clean-up procedure, pH adjustment to extract polar degradation product, the type thin-layer plate and the developing tlc solvent system are dictated by the chemical nature of the xenobiotic. Authentic standards of the xenobiotic, both labeled and non-labeled, are co-chromatographed with the experimental extract. Use a tlc plate containing a fluorescent indicator and locate the compounds by viewing under short wave ultraviolet light (2537A).

Autoradiography. Use no-screen medical x-ray film to locate and visualize the radioactive material by autoradiography. Expose the x-ray film for 7 days to the tlc plate. Identify the radiogram by superimposing the film on the tlc plate. Quantify separated radiolabeled material by carefully scraping silica gel from the specific radioactive R_f area into a scintillation vial containing 15 ml of toluene: fluor (Fluoralloy®) and assay the mixture with a liquid scintillation

counter. Correct all results for background and quench.

Analysis of Results. Report all samples in terms of degradability of xenobiotic, percentage of degradation and chromatographic identification of degradation products. Indicate the extraction solvent, extraction efficiency and the tlc developing system.

Sampling

All samples are taken in replicates of 3 to 5 and expressed as mean values ± standard error; however, confidence interval reference and the chemical nature of the xenobiotic may necessitate a larger sample size. Data are not utilized when mortality within the experimental group exceeds that in the control by 5%.

Water Quality

Water should be uncontaminated and constant quality and should meet the following specifications:

1. Suspend solids, 20 mg/1;
2. TOC or COD, 10 mg/1;
3. Un-ionized ammona, 20 μg/1;
4. Residual chlorine, 3 μg/1;
5. Total organophosphorus pesticides, 50 ng/1;
6. Total organochlorine pesticides plus PCB's, 50 ng/1.

Water is considered to be constant quality if the monthly ranges of the hardness, alkalinity, specific conductance TOC or COD, and salinity are less than 10% of the respective averages and if the range of pH is less than 0.4 unit. Alternative freshwater should be obtained from an uncontaminated well or spring if possible; only as a last resort should a dechlorinated water be used. If dechlorinated water is used, it must be shown that either first instar daphnids can survive in it unfed for 48 hours or that residual chlorine must be measured below 3 μg/1 at the beginning of the test (Stephan, et al., 1975).

Uptake From Water by Microorganisms

Determination of Equilibrium Concentration

Test Organism: Yeast (*Saccharomyces cerevisiae*)

Culture Preparation. Accurately weigh 1.0 g of dried commercial grade baker's yeast and pour it directly into a 2 l beaker containing 1 l of water warmed to ca 48C. Place the yeast suspension on a magnetic stirrer and mix slowly for 1 hr.

Xenobiotic Inoculation and Incubation. After 1 h incubation, prepare yeast cell dilutions of 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml in triplicate 100-ml volumes and place in 250-ml Erlenmeyer flasks. Add the ^{14}C-labeled xenibiotic at the pre-selected concentration directly to each dilution of the yeast suspension. For best results, the total counts per minute (cpm) for the specific radioactive substance used in the uptake segment should exceed 1000 cpm for each sample. Water is the carrier solvent of choice. If a carrier solvent other than water is necessary to introduce the xenobiotic, it should not exceed 0.1% of the total volume of the yeast suspension. The carrier solvent and amount used must be clearly defined in the final report. Incubate the yeast suspensions in the ^{14}C-xenobiotic solution for 0.5, 1, 4, 8, and 24 h in a water bath shaker maintained at 20±1°C. The yeast cells should be moderately agitated to keep them in suspension.

Residue Analysis—Yeast. After incubation for various intervals, vigorously shake the yeast suspension and accurately pipette 50.0 ml of the treated yeast cells from the 250 ml exposure flask for residue determination. Remove the yeast cells from the treatment solution by centrifugation or filtration. Any centrifuge that can exert ca 10,000 × g force is satisfactory. Filtration can be accomplished with a Millipore® type apparatus with a 0.45 μ filter. Wash the cells once by resuspending them in 50 ml of clean water and reconcentrate them by centrifugation or filtration. Immediately add 6 ml of Triton-X 100®:Fluoralloy® (2:3 v/v) to the cells and stir vigorously with a vortex mixer. Pour the yeast suspension directly into a liquid scintillation vial. Wash any remaining cells from the centrifuge tube and filter into the vial with 9 ml of Fluoralloy®-toluene mixture. Place the vial directly into a liquid scintillation counter with an internal standard. Prepare a control sample with untreated yeast cells and the emulsifier-fluor to correct for background radiation. Correct for quench by preparing a ^{14}C-spiked yeast sample at a concentration similar to the one used for the uptake experiments. Convert corrected cpm unit/sample into weight unit of the ^{14}C-xenobiotic by dividing the corrected known specific activity of the compound.

Analysis of Results. Report residue on the xenobiotic yeast cells (wet) (w/w) (convert all dry yeast weights [i.e. the beginning yeast weight] to wet weights; yeast contain ca 50–60% water). All data should represent true replications of samples. Report yeast cell residues as mean ± standard error. Prepare a chart and report the total cell residue for each biomass-incubation concentration. Indicate whether uptake reaches an apparent equilibrium concentration between the yeast cell and the xenobiotic. State the specific time and biomass. Select the biomass and incubation period with the highest residue in the daphnid feeding experiment to demonstrate the maximum movement of the xenobiotic by yeast cells. Obtain the uptake factor (UF) for this value. Calculate this value by dividing the concentration of the *original* water concentration introduced into the culture by the final total cell residue (total cell residue/original H_2O concentration). For example, if the H_2O concentration was 100 ng/l (ng/kg) and the final yeast residue was 10 ng/mg, the UF would be 100,000 times (See Water Uptake Index).

Uptake From Water by Daphnids

Determination of Equilibrium Concentration in Daphnids

Test Organism: *Daphnia magna*

Culture. The filter-feeding cladoceran *Daphnia magna* Strauss is easily maintained in the laboratory in large vats (>50 gal) or in smaller aquaria (<10 gal) feeding only on yeast cell suspensions. The stock culture should be healthy, vigorous and reproducing parthenogenetic with no signs of ephippial "sex".

When collecting daphnids from water avoid unnecessary handling and injury to these fragile organisms by constructing a small hand net. We recommend using small fish net, remove the netting and replace it with #20 wire mesh (or equivalent). Secure the mesh to the outer net rim with builder's sealant (Dow Corning® 781). The daphnids are easily collected on the mesh and then transferred to the experimental chamber or filter pad.

Experimental Chamber. The daphnids are netted from the stock culture, washed with clean yeast-free water, collected on a Millipore®-filter holder containing a No. 2 Whatman-filter paper to remove the wash water and quickly weighed on an analytical balance to the nearest 0.1 mg. One gram (wet weight) of the daphnids are quickly placed in a 2 l all glass aquarium containing 1 l of water. The daphnid sample should contain various ages of instar development.

The aquarium contains a center drain and standpipe with a tubular glass sheath. The glass sheath is notched at the bottom to channel water from the bottom of the aquarium. This drainage system insures better mixing of the introduced chemical and more efficient removal of waste products. Temperature is maintained at a constant 20±1 C. Set photoperiodicity for a 12 hr cycle with fluorescent lighting. All organisms are acclimated to the same water source and temperature used in the uptake experiments. Daphnids introduced into the experimental chamber are acclimated overnight. If the organisms are not freely swimming about the aquarium or if mortality exceeds 5% of the total daphnid population, the experiment should be terminated. The biomass:water volume should not exceed 1 mg daphnid wet weight/1 1 of water.

Xenobiotic Exposure. To insure a relative constant water concentration of the preselected xenobiotic, use a metering pump or a single cell diluter (Chandler *et al.*, 1974) to introduce an intermittent flow at a rate of 2 to 5 ml/min. Excessive flow rate of water in a small aquarium is harmful to the daphnids. The daphnids are not fed during the uptake experiment. Terminate the experiment after 48 h to preclude potential nutritional stress within the culture.

Residue Analysis—Daphnids. Determine total body and water residues at 1, 4, 8, 24, and 48 h to ascertain uptake kinetics. Remove the daphnids from the experimental chamber, wash them with clean water, collect them on a Millipore® filter (No. 1 Whatman filter 4.25 cm) and weigh wet. Homogenize the sample in a tissue grinder with a Teflon®-coated tissue grinder and 6 ml of Triton-X 100® and Fluoralloy® (2:3 v/v emulsifier:fluor). Pour the homogenate into a scintillation vial, washing the grinding vessel with 9 ml of the fluor. Place the vial in a liquid scintillation counter and determine counts per minute present in the sample. Quench and background radiation corrections must be made before calculating specific quantities of xenobiotic present. Accuracy data derived from spiked daphnid samples should also be included with final liquid scintillation analyses. After these appropriate corrections are made, report all residues as ratio of xenobiotic to whole body wet weight of daphnids (w/w).

Residue Analysis—Water. For water residue analysis use a similar experimental chamber without daphnids, but one that is receiving the same xenobiotic concentration and flow rate as the one containing the test organisms. *Do not* use the experimental chamber containing the daphnids because they tend to give a misleading low value due to the uptake by the daphnids. If this residue value must be used, it should be clearly indicated when describing the uptake factor. Take a water sample of sufficient volume to give a minimum of 100 cpm (corrected) per counting vial. Water samples are monitored during the uptake experiment at each daphnid residue sampling interval. Take all water samples in triplicate.

Each water sample is extracted with the most appropriate solvent for that specific chemical. It is strongly advisable to run ^{14}C spiked samples to determine extraction efficiency for the specific xenobiotic-solvent combination. Use an appropriate size separatory funnel which permits good solvent: water partitioning. Avoid vigorous shaking that produces unmanageable emulsions. After extraction, carefully evaporate the solvent to approximately 1 ml in a porcelain evaporation dish under a fume hood. The type of solvent will dictate whether heat or nitrogen is necessary for evaporation. In some instances a rotary-vacuum evaporator may be necessary to reduce the solvent volume. Wash the concentrated solvent directly into a liquid scintillation vial with 15 ml Fluoralloy®-

toluene solution. To reduce phosphorescence store the samples overnight in a dark place prior to placing in a liquid scintillation counter for assay. Correct counts for quench and background radiation with control ^{14}C spiked samples. Water extraction efficiency data should be presented with final residue values. Caution: Certain common solvents used for chemical extraction cannot be used in liquid scintillation spectrometry because of excessive quenching. The investigator is advised to check a manual for preparations of samples for liquid scintillation counting prior to making water extractions.

Analysis of Results. Take all samples in at least triplicate and on a weight/weight basis. All daphnid residues are based on wet weight only. Plot uptake factors (UF daphnid residue/water concentration) for each interval from 1 h to 72 h. Determine the possible plateau concentration for the daphnid at the experimental concentration.

TRANSFER VIA NATURAL FOOD

MICROORGANISMS—FILTER-FEEDER

Organism: Yeast (*Saccharomyces cerevisiae*)

Culture. (See Uptake from Water—Daphnids)

Xenobiotic Exposure. Accurately weigh 2.0 grams of daphnids (wet) and place them in at 2 l glass aquarium containing 1 l of water. The daphnid sample should contain a random collection of various ages of instar development. The chamber is similar to the container used to determine direct uptake of xenobiotic from water by daphnids (Water Uptake—Daphnid). Maintain the daphnids at 20±1°C throughout the feeding exercise. Do not subject the daphnids to any drastic water or temperature difference from the stock culture during the feeding for 2 days before beginning the experiment.

Prepare a yeast culture containing a residue of at least 80% of the previously determined equilibrium concentration (see yeast uptake protocol). Use a 3 l Erlenmeyer flask and prepare a 2 l solution containing a 250 μg/ml concentration of treated yeast. Place the flask on a magnetic stirrer to keep the yeast cells in suspension and attach a metering pump. Pump the treated yeast cells directly into the daphnids at a rate of 1 ml/min. After 24 h, prepare another flask containing a similar concentration of treated yeast cells and replace the orginal flask with this batch for the final 24 hr feeding period. We found that by following this procedure it is much easier to monitor the residue of treated yeast cells during the last 24 h interval.

Residue Analysis. Remove three 50-ml samples from both the 24 h and 48 h yeast batch and determine the total cell residue of the xenobiotic passed via the microbial food route. Yeast residues are determined by the liquid scintillation method (See Uptake from Water—Microorganisms—Residue).

After 48 h remove all the daphnids from the feeding chamber. Wash the daphnids with clean water to remove any adhering yeast cells. Take at least five replicate samples of daphnids from each experimental chamber. Each sample should contain at least 100 mg of daphnids (wet weight) for residue analysis. Each feeding experiment should be set up in at least duplicate.

Analysis of Results. Report all residue data on a wet weight basis. Calculate and report the transfer rate of the xenobiotic from yeast to daphnids on a percentage or ratio basis (total daphnid residue/total yeast residue) (Also see Food Transfer Index).

FILTER-FEEDER—FISH

Organisms: Daphnids (*Daphnia magna*)
Bluegill (*Lepomis macrochirus*)

Natural Food Preparation. Daphnids should contain a residue of the xenobiotic at concentrations at least 80% of the equilibrium concentration (previously determined in the Daphnid-Uptake Section). Generally the ^{14}C-labeled xenobiotic may be conserved by introducing the radioactive material into the daphnids in a static system, instead of a flow-through apparatus. Take 2.0 g (wet weight) of daphnids from the stock culture by screening and place them into a 2 l beaker containing 1 l of water. Add the chemical directly to the beaker container 1 l of water and expose the daphnids overnight. The concentration of the xenobiotic employed usually is higher than those used to determine the equilibrium concentration. Start with a water concentration about five times that used in the plateau experiment. In most cases a few trial tests will be sufficient to determine when 80% of the equilibrium concentration is reached. If a carrier solvent is necessary to introduce the xenobiotic to the daphnid, it should not exceed 0.1% of the total volume of the water containing the daphnids. Also, the static concentration selected for exposing the food source should not exceed the LC50 value of the xenobiotic for the daphnids. The treated daphnids are removed from the exposure solution by filtration. Use a Millipore® stainless-steel filter holder with a 4.25 cm No. 2 Whatman filter paper. The wet, live organisms are quickly weighed to the nearest 0.1 mg. Avoid prolonged filtering and air-drying to reduce potential injury to the daphnids prior to feeding them to fish. The number of daphnids needed for each feeding is estimated from the composite fish weight of each group. Feed the bluegill an amount of daphnids equivalent to 5 to 10% of the fish weight per day. Other rations may be selected, but the total amount of live food given each group per day must be clearly delineated. After the treated daphnids have been weighed, divide them between each group of fish on a 5 to 10% per fish weight per day. For example, if the fish in group 1 weigh 3500 mg (composite weight) and those in group 2 weigh 3700 mg, the daphnid ratio are 350 mg and 370 mg, respectively. Normally, the daphnids are completely consumed within 30 min by the fish. If the live food is not taken within this interval, we suggest that the daily ration be divided into two or three smaller feedings during each day.

Residue Analysis—Food. Total body residues of the daphnids are determined for each feeding. Take duplicate 50-mg aliquots of the composite daphnid feeding for residue analyses. After weight determination, mix the sample with 6 ml of Triton-X 100: Fluoralloy® (2:3 v/v) emulsifier:fluor in toluene and homogenize with a teflon-coated Sorvall tissue grinder. Transfer the homogenate into a glass scintillation vial, bring it to a 15-ml volume with 9 ml of the fluor-toluene mixture and count overnight in a liquid scintillation counter. Make correction for quench and background radiation. Determine accuracy values with spiked ^{14}C samples.

By carefully monitoring the residues in the daily feeding throughout the 30-day interval, any adjustment in the daphnid fed the fish can be made.

Experiment Chamber—Fish. The fish tanks are all glass and have a 78.6 liter (20 gal) capacity. Each tank has a central drain and stand pipe with a PVC sheath (See Uptake—Daphnids) to insure proper removal of waste products from the lower layers of the container. The flow rate to each tank is adjusted for three complete turnovers per 24 h. The experimental tanks should be located in areas away from non-essential traffic. Every effort should be made to shield the fish from unnecessary visual and mechanical stimuli. Expose the fish to normal light and dark sequence from a natural source or use a 12 h photoperiod cycle with fluorescent light. Overcrowding of fish must be avoided. Fish loading per aquarium must not exceed 1 g fish per 1 l water. Water is maintained at 20±1°C. Acclimate the fish to the test chamber, temperature and intake of natural food for several days prior to the beginning of the experiment. Abrupt changes in temperature and water quality are to be avoided. Monitor each test tank for temperature, ammonia and oxygen concentration throughout the 32 day feeding schedule to detect and prevent potential stress of the fish (See also Stephan, et al., 1975).

Use bluegill (*Lepomis macrochirus*) preferably within the 0.5 to 1.5 g range. Avoid marked aggressive behavior and unequal food consumption between fish during the feeding experiments by carefully selecting fish on the basis of similarity in size and weight. The fish should be vigorous and healthy; the bluegill should readily accept natural food after acclimation. Disease or mortality in the holding stock should be negligible. The investigator should determine that all materials which may come in contact with the fish are nontoxic.

Fish Feeding Procedure. Distribute 50 bluegills equally into five 78.6 liter (20 gal) tanks. The five tanks with 10 fish each are designated groups 1, 2, 3, 4, and control. Groups 1, 2, 3, and 4 are fed treated daphnids for 7, 14, 21, and 28 days, respectively. The control is fed untreated daphnids for 28 days. The feeding schedule proceeds as follows:

Table 1—Daphnid-Bluegill Feeding Schedule
Duration: 32 days

Day:	*Activity*
0	Determine fish weight, groups 1, 2, 3, 4; Estimate daphnids biomass
1–2	Fish acclimation to aquaria
3–9	Feed daphnids to fish, groups 1, 2, 3, 4; daphnia TBR [1] determined daily
10–11	Clearing (no feedings) and determine TBR group 1; feeding adjustment [2], groups 2, 3, 4
10–16	Feeding Groups 2, 3, 4; daphnid TBR determined daily
17–18	Clearing and TBR group 2; groups 3, 4 feeding adjustment
17–23	Feeding Groups 3, 4; daphnid TBR daily
24–25	Clearing and TBR group 3; feeding adjustment group 4
24–30	Feeding Group 4; daphnid TBR daily
31–32	Clearing and TBR group 4; Experiment terminated
Optional	Group 5 degradation and/or clearing study

[1] Total body residue
[2] See text

Residue Analysis—Fish. When the specific feeding period is completed, the group receives no food for 48 h. After the 48 h elimination period, which permits the digestive tract of the fish body residue loss to evacuate, each fish from the groups is removed,

blotted dry and weighed. Each fish is ground *in toto* in a tissue grinder with 6 ml of Triton X–100 Fluoralloy® (2:3, emulsifier:fluor, v/v). Quantitatively transfer the homogenate from the griding tube with 9 ml rinse of the fluor-toluene mixture to a liquid scintillation vial. Because of potential quenching action with fish tissue, the total fish biomass per vial should not exceed 500 mg. If the sample is large, divide the sample into several subsamples and combine the counts for the final total body residue determination. Keep the liquid scintillation samples in the dark for at least 24 h to preclude counting errors due to internal phosphorescence. Place the samples in a liquid scintillation counter and determine counts per minute (cpm) for each sample. Convert cpm into quantities of xenobiotic present in each fish sample.

Analysis of Results. Report all residues on a weight/weight basis. Use only the wet weight values. Express all data as the mean ± standard error. Report fish residue values for the 7, 14, 21, and 28 days. Prepare a food transfer index for the xenobiotic (Total fish residue/Total daphnid residue). (Also see Index).

Uptake From Water and Natural Food

Combination Studies

Organisms: Daphnids (*Daphnia magna*)
Bluegill (*Lepomis macrochirus*)

Determine the uptake of xenobiotic by fish directly from water, from live food, and from a simultaneous combination of the two. The protocol previously describing the transfer of xenobiotic via natural food to fish closely parallels this procedure with one exception, the xenobiotic is also introduced directly to the fish from water.

Experimental Chamber. Select at least four 78.6 l all glass aquaria with central drains and stand pipe with PVC (see also Water Uptake—Daphnid). Place 40 fish in each tank (see fish selection in feeding section) and maintain at 20±1°C. Use the McAllister *et al.* (1972) chemical metering device with the Mount and Brungs (1967) flow-through apparatus to deliver a pre-selected concentration of the xenobiotic.

Food Transfer and Xenobiotic Exposure. The four tanks are designated "control", "food" (F), "food and water" (F&W), and "water" (W). The "control" and "food" tanks receive only the carrier solvent of the xenobiotic from the diluter effluent. Tanks labeled "food and water" and "water" receive the xenobiotic in the effluent diluter flow at a predetermined concentration. These two tanks receive the xenobiotic after passing from a connecting mixing box (Benoit and Puglisi 1973). This process assures relative equal exposure concentration between the two test groups. The flow rate from the diluter should result in at least 3 complete turnovers/aquarium/24h. Tanks marked "food" and "food and water" receive treated daphnids at a rate of 5–10% fish weight per day (See daphnids feeding protocol). Feed the tank marked "control" and "water" untreated daphnids on a similar percentage basis. Take water samples in triplicate to verify the xenobiotic concentration introduced into the "water" and "water and food" tanks. Remove water samples daily for the first three days. If the water concentration of the xenobiotic remains relatively constant, take water samples twice per week thereafter (the fish residue day and four days later). Take water samples directly from the diluter effluent prior to its introduction into the experimental chamber with fish. (Water analysis—see Water Uptake—Daphnids).

Fish Feeding Procedure. (See Food Transfer—Filter-Feeding—Fish)

Residue Analysis Daphnid—Fish (See Food Transfer—Filter-Feeding—Fish)

Analysis of Results. All sample residues are expressed on a weight/weight basis. Express fish and daphnid residues only on a wet weight basis. All data are expressed as mean ± standard error. Report the mean water residue per week. Prepare a graph showing the uptake of xenobiotic by fish from water, food and the combination of both over time. Calculate an uptake factor (total body residue/water concentration) and uptake index (residue in fish/residue in food) (See also Index).

Water Uptake and Food Transfer Index

The food transfer index and water uptake index are both based on the comparison of a xenobiotic uptake directly from water or transfer from one trophic level to another via natural food with a reference compound. Because of its ubiquity and persistency in the natural environment, p,p'-DDT [1,1,1-trichloro-2,2 *bis* (p-chlorophenyl) ethane] is recommended as a reference xenobiotic Food Transfer Index. For example, if a bluegill contains a mean DDT total body residue (TBR) of 2 mg/kg after feeding 30 days on daphnids containing a mean TBR of 1 mg/kg DDT, we calculate a food transfer factor (FTF) as follows:

$$\text{FTF}=\frac{\text{Fish TBR}}{\text{Daphnid TBR}}\ \text{(w/w, wet weight)}$$

Substituting

$$\text{FTF}=\frac{2\ \text{mg/kg}}{1\ \text{mg/kg}}$$

$$\text{FTF}=2.0$$

(DDT)

If compound "Q", an experimental pesticide is introduced into a similar system and the daphnids contain a compound "Q" TBR of 250 μg/kg and the fish after the 30-day feedings contain a TBR of 50 μg/kg Compound "Q", the FTF is 0.25

$$\left[\text{FTF}=\frac{50\ \mu\text{g/kg}}{250\ \mu\text{g/kg}}\right]$$

Calculate the food transfer index as follows:

$$\text{Food Transfer Index}=\frac{\text{Unknown Food Transfer Factor}}{\text{Reference Food Transfer Factor}\times 100}$$

Substitute the above data:

$$\text{FT Index}=\frac{0.25}{2.00}\times 100=12.5$$

$$\text{FT Index}=12.5$$

(Compound "Q") (DDT=100)

Water Uptake Index. Similarly a water uptake index may be prepared. First determine the uptake factor (UF) of the xenobiotic from water. Calculate this factor by dividing the water concentration of the xenobiotic (exposure concentration) into final daphnid TBR (preferably at an equilibrium concentration). For example, if the daphnids contain a TBR of 10 μg/mg p,p'-DDT after exposure to a 100 ng/l solution, we calculate the UF as follows:

$$\text{UF}=\frac{\text{Daphnid TBR}}{\text{H}_2\text{O concentration}}\ \text{(wet weight)}$$

Substituting

$$\text{UF}=\frac{10\ \mu\text{g/mg}}{100\ \text{ng/kg}}$$

$$\text{UF}=10{,}000\ \times$$

If in a similar system the UF value for daphnid exposed to compound "Q" is 5,000 x, we may calculate a Water Uptake Index (WU Index) as follows:

WP Index=unknown UF/Reference UF×100
Substituting:
WU Index=5,000/10,000×100
WU Index=50
(Compound "Q") (DDT=100)

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Field-Testing Techniques [1]

No universally applicable methods are available for field testing of pesticides because of the wide diversity of mechanisms whereby a pesticide may enter the environment, the diversity of habitats which may be affected and the nature of the pesticide (solubility in water, degradability, etc.). However, a discussion of some aspects of field testing and a documentation of some previously used methods should be a useful aid to investigators in designing tests to meet particular situations. The following discussion is derived principally from a paper by Livingston et al. (1974) and was prepared by Dr. J. L. Lincer of the Mote Marine Laboratory.

Toxicity Tests Versus the Field Approach

The objective of laboratory toxicity tests is to determine the effect of a chemical on organisms under defined conditions. Toxicity tests are normally made under controlled conditions with only one or two variables, one normally being the toxicant. Often un-

[1] For a more comprehensive discussion of this topic, see Livingston, et al. (1974).

natural conditions are imposed upon the organisms being tested or effects which are important in the field are not considered. In acute tests, for example, the organisms are not usually fed nor are they permitted to avoid the toxicant, a behavior which may be possible in the field. The exposure period is for 1–4 days, often under static conditions, and the toxicant is not diluted as it normally is in the field. Chronic tests may start to approach the real world but, still, destructive or removal mechanisms, like sorption to particulates or photo-oxidation, are usually not present in the laboratory tests. Perhaps most important is the fact that organisms are usually not exposed to only one toxicant in the field. Laboratory tests have an obvious place in determining the toxicity of a chemical to various organisms or the toxicity of various chemicals to a given organism under specified conditions. However, extrapolation to field situations is not always feasible.

Although there is reason to believe that carefully controlled laboratory and field results may coincide (Mount, 1974), proper final diagnostic evaluations of effects must be made under adequately monitored field conditions with assist from carefully designed toxicity tests. Such studies should be interdisciplinary and are expensive but, in the end, probably provide the only definitive results.

SPECTRUM OF TECHNIQUES

In general, to find the ecological relevance of a laboratory finding, one moves in the direction of field studies. To find the cause(s) for an observed disturbance of the environment, it is often necessary to move into more controlled laboratory situations. Table 1 provides an overview of the kinds of ecosystems, pesticides, observed parameters and taxa reported in the literature. Table 2 displays aquatic species (by broad taxa) observed and/or used in field-testing pesticides.

Field Studies. In a *field survey*, a sampling method is devised to include: a broad range of the animal and plant life, both perturbed and unperturbed areas, seasonal changes, and where possible, before and after effects of some event, such as the application of a potentially registrable pesticide.

In contrast, a *monitoring program* implies continual sampling of the environment in order to detect changes as they occur. Depending on the circumstances at each site, it may be appropriate to shift from the survey to the monitoring program, or vice versa.

Alternatively, in *field tests*, organisms are maintained in cages or confined areas in the field environment. Such systems can continually assess the effect of the application on a series of representative species.

Aside from the physical difficulties of sampling or monitoring at particular sites or seasons, there are other potential disadvantages. Such methods can show that changes have taken place in organisms or in communities of organisms, but do not show what *caused* the changes unless adequate consideration is given to the statistical approach. At the very least, suitable replications and control areas are a must, and, of course, the value of pre-application field data becomes obvious.

Perturbed or Partitioned Environments. A portion of the real world can be partitioned off and purposely contaminated. Problems with this type of research are: (1) Deciding whether deliberate damage to even a small portion of the environment is justified by the information that will be obtained, (2) confining the damage to the area under study, (3) deciding whether the portion of the environment under study is representative, and (4) achieving sufficient control over the test area. The advantages are that the spill or contamination is under the control of the investigator so that pre-stress data can be gathered and the actual stress manipulated and measured, while the complexity of the real world is retained to a greater degree than in a laboratory study.

Models. Laboratory streams are full-scale models, over which a greater degree of control can be exercised than in a partitioned and perturbed stream in nature. Since the investigator has created an environment, he does not have the ethical problem of deciding whether his ends justify purposeful damage to a portion of the natural environment.

Microcosms are greatly simplified ecosystems, with perhaps less than ten species to represent some of the interaction occuring among hundreds of species in the environment.

Odum and others have developed methods of simulating ecosystem energy and materials flows on analog *computers*. Waller, et al. (1971) developed a stochastic model and computer program to predict long-term effects of different environmental zinc levels on as many as 100 generations of fat-head minnows. The predictive capability of such abstract models can often be improved as additional field and laboratory data become available. On the other hand, such models often indicate what types of information need to be gathered to understand ecosystem responses to perturbations. The parameters in stochastic models are allowed to vary, which probably better approximates the real world, than a nonvarying model.

Models have the advantage that the effect of environmental manipulations, even extreme manipulations, can be tested through many generations or seasonal cycles, without damage to the real world. The disadvantage of such models is that they are *twice removed* from reality—the model is a construct or simplification based on a sampling of information about the real world.

In summary, there are advantages and disadvantages associated with any technique used to determine pesticidal effects. Techniques should be used which combine elements of both field and lab approaches. The best problem-solving strategy is to use the techniques that will best answer the questions being asked, and to avoid rigid adherence to one technique.

FEASIBILITY OF FIELD TECHNIQUES

With the inherent difficulties of sampling and variability encountered in any field study it might seem futile to attempt any such evaluation. If it is possible to adequately describe some aspect of a community then it should also be possible to identify changes in this characteristic induced by potential stressors, such as pesticides. The fact that this has *not* been done with any degree of sophistication arises from our inability to satisfactorily describe the unit we are dealing with.

Problems in studying a single species under field conditions revolve around sampling difficulties associated with the particular species. These difficulties may be minor with small randomly distributed sessile animals but great with mobile species and species with non-random distribution patterns. Nevertheless, it is still much easier to monitor changes in the growth rate, survival rate or reproductive activity of a single species than it is to evaluate a community or species assemblage.

The alternative to the community parameter approach is to consider elements of the fauna on an individualistic basis; identify and count the species collected and try to develop some understanding of the biology involved. This takes much more effort but the information return is much more useful. Unfortunately, there are no rules which have general application to all situations. Different communities and localities may require different sampling procedures. In some cases, dominant species may have a profound effect on the biology of a community and the fate or responses of *these* species (or opportunistic species that replace them) may provide useful predictions as to what might happen to the community as a whole.

Most applied environmental work reported in the literature has been executed in such a manner that it would detect only relatively major changes. However field approximations of pollutant interactions can be of paramount importance. Koenig, et al. (1974), for example, found that blue crabs (*Callinectes sapidus*) contaminated with DDT and its metabolites did not die in the field from such contamination unless periodic cold fronts caused significant reductions in the water temperature. The combined effect of the pesticide and temperature stress caused significant mortality. In this way field observations allowed identification of pollutant impact as a result of interaction with a significant modifying factor. This would emphasize a need for further experimental and field work on the impact of various pollutants under conditions of natural stress.

I spite of major difficulties, it is expected that as the theoretical and practical aspects of environmental research improve, there will be a capability to measure the effects of single and combined factors under field conditions. However, *"at this point in time"*, it appears that the only possible approach that might be standardized is that of the cage-type or confined-area exposure (Table 3). Since the confined area type of study requires a high degree of knowledge of the life history of the various species, the ecology of the area, etc., this would be very difficult to reduce to a routine.

GENERAL PRECEPTS FOR CAGE-TYPE FIELD-TESTING

Acquiring Organisms. Organisms may be collected locally or purchased from commercial fishermen. If captured, acceptable techniques, such as those recommended by the Environmental Protection Agency (1973), should be used. Whether organisms are collected directly or purchased, every effort should be made to insure that they are healthy and are not subjected to unnecessary stress. Perkins (1972) discussed the importance of using healthy animals and emphasizes the importance of stresses such as collecting, handling and maintenance.

Parameters. Parameters that should be studied which would relate to individual species include:

Mortality
Growth
Reproductive Success
Gross Behavior

Other observable changes within such fields as physiology, biochemistry, pathology, etc., which relate *directly* to the survival of the species.

Test Organisms. Based on the literature reviewed to date (Table 3), the *most common* species of fish used in cage-type field-testing pesticides are from the marine environment and are as follows: Sheepshead minnow (*Cyprinodon variegatus*); Mosquitofish (*Gambusia affinis*); Sailfin molly (*Poecilia latipinna*); Striped mullet (*Mugil cephalus*); Mummichog (*Fundulus heteroclitus*).

A similar list for invertebrates would include:

Crabs: Blue crab (*Callinectes sapidus*); Fiddler crab (*Uca minax, U. pugnax*).

Shrimp: Glass (*Palaemonetes pugio*); Other species of shrimp (like *Penaeus spp.*) are readily available, but have not been used frequently in cage studies.

Mollusks: Oyster (*Crassostrea virginica*).

Cage Construction. The construction of cages for specific taxa depends, of course, on the species, its predators, the habitat and the

physical and chemical properties of the pesticide being tested. Although there has been some effort to describe an exposure cage for this purpose (Heitmuller and Nimmo, 1972), most cages vary with the investigator (see Table 3 for such studies).

Introduction of Pesticides and Monitoring. Pesticides should be introduced at the season and using the amount and formulation(s) expected to be used under actual spraying conditions. If adequate safeguards are available, another application at ten-fold the expected level might be applied to help identify subtle effects which could be important. Monitoring of the residue levels in the organisms and their environment over time is of particular importance with persistent pesticides. Water quality parameters (e.g., pH, temperature, and conductivity at least) should also be regularly monitored.

The Test Area. The area to be used should be as homogeneous as possible with respect to the biotic, physical and chemical environment. If the conditions change over time, they should be definable in terms of periodicity. Every effort should be made to choose an area which allows the investigator to prevent, control or minimize the spread of the applied pesticide.

Some General Considerations. 1. Be realistic in choice of species. Species collected locally will normally be easier to work with.

2. Be aware of the possibility of induced resistance.

3. If caged animals are to be used, a reasonable period of acclimation is necessary.

4. Within the constraints of acceptable techniques, choose the most sensitive species and/or life stage inhabiting that ecosystem.

TABLE 1.—*An overview of the field-testing of pesticides*

Ecosystem	Pesticide	Observed parameters	Taxa and/or species	Reference
Freshwater pond	DDT	Mortality	Fish, plankton, benthic invertebrates, reptiles, birds, mammals, terrestrial insects.	Tarzwell, 1948.
Enclosed area of freshwater pond.	DDT	Population	Fish	Do.
Tidal marsh ditch	DDT	Mortality and population; residue monitoring.	Fish, crabs	Croker and Wilson, 1965.
Do	Dieldrin	Mortality	do	Harrington and Bidlingmayer, 1958.
Tidal marsh	Strobane, DDT, and HCB	Mortality, gross behavior on fiddlers.	Fish, crabs, birds, mammals	George, et al., 1957.
Do	DDT. Aldrin, Dieldrin, and BHC.	Mortality	Fish, prawns, arthropods, isopods and amphipods, crabs, worms, mollusks, birds.	Springer, 1961.
Tidal marsh	DDT	Mortality, gross behavior growth (snails).	Fish, crabs, shrimp, insects, mollusks, amphipods, worms, mites, birds.	Springer and Webster, 1951.
Do	Dursban	Mortality, cholinesterase inhibition.	Fish, crabs, shrimp, mammals, birds.	U.S.D.I., 1968.
Mangrove swamp	Dibrom	Mortality, cholinesterase population.	Fish, crabs, mammals, birds, insects.	U.S.D.I., 1967(b).
Salt marsh, freshwater pond	Dursban	Mortality	Fish, shrimp, crabs, oysters, insects, terrestrial vertebrates.	U.S.D.I., 1967(a).
Estuaries	2,4-D	do	Fish, crabs, oysters, clams	Rawls, 1965.
Salt marsh	Malathion	Mortality, cholinesterase	Fish, crabs, shrimp, molluska	Tagatz, et al., 1974.
Do	Dursban	Mortality, monitoring	Fish, crabs, shrimp, birds	Ludwig, et al., 1968.

TABLE 2.—*Aquatic species observed and/or used in field-testing of pesticides*

Pesticide	Fish	Crabs	Shrimp	Other invertebrates	Reference
DDT	Striped mullet, sheepshead minnow, rainwater killifish, sailfin molly, mosquitofish, spot, longnose killifish, gulf killifish, tidewater silverside, diamond killifish, darter goby.	Blue crab, fiddler	*Palaemonetes* species	Annelid worms	Croker and Wilson, 1965.
Dieldrin	Ten pounder, sea catfish, sailfin molly, striped mullet, snook, Irish pompano, red drum, spot, sheepshead, etc.	Portunids xanthids, fiddler, blue crab.	Shrimps	Amphipods, nudibranchs, snails, tethyonids, oysters.	Harrington and Bidlingmayer, 1958.
Strobane, DDT and BHC.	Mummichog, sheepshead minnow, spot, white mullet.	Blue crab, marsh fiddler, red-jointed fiddler, marsh crab.		Bryzoans	George, et al., 1957.
DDT, Aldrin, BHC, Dieldrin.	Various fish	Blue crab, marsh carb	Prawns	Isopods, amphipods, arthropod, molluska (including snails and mussels), clam worms.	Springer, 1961 (abstract).
DDT	Mummichog, sheepshead, minnow, *Fundulus*.	Blue crab, fiddler	Grass		Springer and Webster, 1951.
Dursban	Pinfish, sheepshead, drum, mollies, *Fundulus*.	do	Bait		U.S.D.I., 1968.
Dibrom	*Fundulus*, snook Mangrove snapper.	do			U.S.D.I., 1967(b).
Dursban	Various species	do	White		U.S.D.I., 1967(a).
2,4-D	Pumpkinseed	Blue crab		Oyster, soft shell clam	Rawls, 1965.
Malathion	Sheepshead minnow	do	Grass pink	Snail	Tagatz, et al. (1974).
Dursban	Croakers, killifish, mullet	do	Brown		Ludwig, et al., 1968.

TABLE 2.—*Aquatic species used in field caps (C) of confined area (CA) type studies*

Species	Type of study (C or CA)	Reference
Fish:		
Sheepshead minnow (*Cyprinodon variegatus*)	CA	Croker and Wilson, 1965.
Gulf killifish (*Fundulus grandis*)		
Longnose killifish (*F. similis*)		
Rainwater killifish (*Lucania parva*)		
Mosquitofish (*Gambusia affinis*)		
Sailfin molly (*Mollienesia latipinna*)		
Striped mullet (*Mugil cephalus*)		
Tidewater silverside (*Menidia beryllina*)		
Mummichog (*F. heteroclitus*)	C	George, et al., 1957.
"Variegated cyprinodon" (*C. variegatus*)		
Spot (*Leiostomus xanthurus*)		
White mullet (*Mugil curema*)		
Cyprinodon killifish	C/CA	Springer and Webster, 1951.
Fundulus specie		
Pinfish	C	U.S.D.I., 1968.
Sheepshead		
Drum		
Mollies		
Fundulus		
Flounder (*Paralichthys* specie)	C	U.S.D.I., 1967(a).
Mullet (*Mugil cephalus*)		
Puffer (*Sphaeroides* specie)		
Sailfin molly (*Mollienesia latipinna*)		
Sheepshead minnow (*C. variegatus*)	C	Tagatz, et al. (1974).
Mullet (*M. cephalus*)	C	Ludwig, et al., 1968.
Croakers (*Leiostomus xanthurus*)		
Broad Killifish (*C. variegatus*)		
Gulf Killifish (*Fundulus grandis*)		
Invertebrates:		
Fiddler crab (*Uca minax*)	C	Croker and Wilson, 1965.
Blue crab (*Callinectes sapidus*)	C	George, et al., 1957.
Marsh fiddler (*Uca pugnax*)		
Red-jointed fiddler (*U. minax*)		
Marsh crab (*Sesarma reticulatum*)		
Blue crab (*C. sapidus*)	CA	Springer and Webster, 1951.
Marsh fiddler (*U. pugnax*)	C	
"Bait" shrimp (*Palaemonetes pugio*)	C	
Do.	C	U.S.D.I., 1968.
Fiddler crab		
Blue crab (*C. sapidus*)		
White shrimp (*Penaeus setiferus*)	C	U.S.D.I., 1967(a).
Blue crabs (*C. saphidus*)		
Fiddler crabs (*Uca specie*)		
Oysters (*Crassostrea specie*)		
Soft shell clam (*Mya arenaria*)	C	Rawls, 1965.
Blue crab (*C. sapidus*)		
E. oyster (*C. virginica*)		
Blue crab (*C. sapidus*)	C	Tagatz, et al. (1974).
Grass shrimp (*Palaemonetes vulgaris*)		
Pink shrimp (*P. pugio*)		
Snail (*Littorina irrorata*)		
Blue Crab (*C. sapidus*)	C	Ludwig, et al. (1968).

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SUBPART C

Hazard evaluation: Wildlife toxicology VII.C–1
Acute and subacute toxicity studies on wild mammals VII.C–3
Protocol for determination of the approximate maximum tolerated dose VII.C–4
Protocol for laboratory acute oral toxicity—Birds VII.C–8
Protocol for determining lethal dietary concentrations of chemicals to birds (5 day-dietary-LC–50) VII.C–16
Reproductive tests on birds VII.C–21
Protocol for evaluation of reproductive effects of pesticides on the bobwhite VII.C–22
Protocol for evaluation of reproductive effects of pesticides on the mallard VII.C–29
Protocol for laboratory acute dermal toxicity test VII.C–42
Protocol for small pen simulated field test to evaluate pesticide hazards to birds VII.C–51
Protocol for large pen simulated field studies VII.C–54
Protocol for full-scale field tests to evaluate pesticide hazards to wildlife VII.C–72

PART VII—HAZARD EVALUATION

SUBPART C—WILDLIFE TOXICOLOGY

Protocols for wildlife toxicology were compiled from a thorough review of the literature and unpublished data and from consultation with nearly all the wildlife toxicology experts in the field. All available protocols were evaluated and those judged most complete and valid have been included. Points of clarification were made throughout the accepted protocols. In most cases only one protocol was deemed applicable and in others it was necessary to prepare composites of protocols in the literature to arrive at an applicable protocol. The test methods were selected because of their overall strengths, with the recognition that any test method does have weaknesses.

Some philosophical preamble to each protocol has been included. This introduction specifies the validity of the test method.

For wildlife the acute oral toxicity test provides a standard unit of measurement to be compared with those of mammalian toxicity and toxicity to other organisms and, in the case of expected granular use, a direct measurement of effects likely from field applications. The 5-day dietary LC–50 is appropriate in determining the effect of a chemical applied to plant matter in the field. This test allows ingestion of a chemical and degradation as it passes through the digestive tract, absorption and metabolism of the pesticide over several days exposure at lesser concentrations. In addition the 5-day dietary LC–50 test exposes wildlife to compound volatility that may occur from the daily diet and to dermal contact via the face and feet from food in the container or that which is spilled. Together, both tests give information regarding chronicity of a pesticide.

The reproductive test should be used when it is known that the parent compound or one of its metabolites persists at significant levels in the environment for prolonged periods or when wildlife are subjected to repeated or continued exposure. This test provides data on a longer term feeding study than the dietary LC–50 and on reproduction and survival of young wildlife. Logically this test should follow data available from subacute, short-term dietary studies.

When requested under special conditions the acute dermal toxicity test may be requested and should follow the acute or subacute toxicity test(s).

As toxicity tests migrate from the laboratory to full-scale field tests, it becomes more realistic, but more difficult to control important parameters and variables. The simulated field tests, both small-pen and large-pen, provide intermediate data to evaluate wildlife toxicity under semi-natural conditions. Simulated field tests necessarily follow acute and sub-acute toxicity studies. Large-pen simulated tests have been used to measure chronic effects, including those on reproduction.

Data from laboratory tests and simulated-field tests are useful in planning an unrestricted field test. Again by necessity this test must follow both the acute and subacute toxicity tests and generally the simulated field tests. This unrestricted field test gives data on actual commercially treated pesticide target areas where non-target wildlife live unhampered in their feeding, mating and reproductive activities.

ACUTE AND SUBACUTE TOXICITY STUDIES ON WILD MAMMALS

Although general laboratory or domestic mammal toxicity data are required on both active ingredient(s) and formulation(s) as a normal component of the application for a pesticide registration, certain unique conditions of proposed usage may lead to special requirements for single dose (acute) and subacute toxicity studies on wild mammals. While comparative toxicities of compounds can be rather well judged by the results with standard laboratory mammalian tests, the extent of species variations in pesticide toxicity is certainly large. Therefore, it may be of occasional importance to undertake some additional toxicity evaluation with wild species occupying habitats which may be impacted by a proposed usage. Such data should only be required of the actual formulation(s) proposed for use.

The test protocol for determination of the "approximate maximum tolerated dose" develops minimal data regarding a species' sensitivity. It is useful when costs and various other complications in conducting tests with large and/or rare animal species preclude conventional toxicity testing. If these constraints do not apply, acute oral LD–50 and subacute dietary toxicity tests should be undertaken employing standard protocols for mammalian toxicity tests with management appropriate to the test species selected (e.g. Webb and Horsfall, 1967).

PROTOCOL FOR DETERMINATION OF THE APPROXIMATE MAXIMUM TOLERATED DOSE

The purpose of this test in wild mammals is to detect species differences to the toxic effects of a pesticide. As such, it is not always essential that precise LD–50 values be ascertained, rather the approximate maximum tolerated dose is estimated and the character of the toxic syndrome determined. Careful observations and thorough necropsy examination are essential.

Testing is carried out by preliminary range finding to establish the approximate lethal dose, and subsequently dosing additional animals with amounts according to the geometric progression indicated by the results or standard mammalian acute toxicity tests. Testing may use a single and/or multiple dosing schedule. The latter, if extended for at least 10 days or more, can be used to arrive at an impression of the relative chronicity of the pesticide in the wild species in place of an extended subacute toxicity test. After dosing, animals are observed for several days for delayed toxic effects. Beyond simply mortality, signs of intoxication such as loss of weight, anorexia, diarrhea, etc., are important. The general procedures followed in testing the toxicity of herbicides in cattle and sheep by the Toxicological Investigations Laboratory, Animal Disease and Parasite Research Division of the U.S.D.A. Agricultural Research Service are typical and represent common practice (U.S.D.A. Agricultural Research Service, 1969).

METHODS AND MATERIALS

Animal Selection. It must be appreciated that in undertaking toxicological evaluations using test species outside the normal types, many uncertainties arise and standardized protocols for such research do not exist. Those well-known major factors that influence LD–50 values within a species such as genetics, sex, age, nutritional and stress factors relating to unnatural conditions as well as social aspects within the micro-environment, are all subject to poor control and the test results, at best, are subject to uncertain interpretation. These precautions are ably discussed by Hurni (1970). The best policy to follow is to attempt, within the constraints dictated by the particular species selected, to exercise those principles of animal selection and management known to be sound: animals must be in healthy condition, as free of social and environmental stress as possible, acclimated to diet and uninfluenced by drugs. Normally, adults of similar age, as judged at least by body weight (but preferably by additional methods as well), would be used and data from both sexes should be obtained if possible. Use of reared stock would generally be preferable to live-trapped animals.

Animal Conditioning. Animals selected from appropriate sources are allowed a prolonged period of acclimatization to the test facilities and feed. Use of paired controls is desirable in tests with wildlife.

Dose Selection. Dosages are calculated on a mg/kg body weight basis for the active component(s) of each chemical tested. Selection of the dosage rates for each type of test animal involve several variables. The initial dosage level is estimated from existing acute toxicity data and the toxic range found by trial and error. When a toxic dosage was found, additional dosages above and below this rate are applied to other animals. Where a step-by-step increase of dosage indicates increased toxicity, repetition of individual dosages is not considered essential.

Chemical Administration. Oral dosing is generally accomplished by stomach placement of gelatin capsules containing the chemical or its formulation using a balling gun, or by "drenching" in which a solution or suspension of the chemical is introduced into the stomach by syringe and stomach tube. Occasionally it is necessary to conceal the dose in an attractive food item.

Toxicity differences may arise from the manner of dosing. Drenching usually involves water-diluted preparations as compared to undiluted material placed in capsules. Data from the ARS Toxicological Investigations Laboratory indicate general inconsistency between these forms of dosing but no generalization is possible favoring one over the other. Use of a solvent such as corn oil and its volume may also affect the acute toxicity evaluation.

For the immediate purpose of interspecies comparisons, it is suggested that the solvent or dispersant should match that used in the primary mammalian acute toxicity testing. The volume administered orally should never exceed 2–3 percent of the body weight and should be constant at all dosage levels.

Absorption effects related to the amount of feed in the digestive tract are normally minimized by overnight starvation prior to administration of the chemical dose. Feed should be provided following dosing and water *ad libitum* prior to and following dosing. Withholding feed for ruminants serves little purpose and it is preferred to maintain such animals on restricted feed allotments calculated to maintain body weight.

Dosing Schedule and Test Design. Testing may use a single and/or a multiple dose schedule. Using the single dose schedule the test animal is observed for several (approx. 10) days following the dose for signs of toxic effect (loss of weight, anorexia, abnormal function or behavior). Using the multiple dose schedule the test animal is observed during a series of 10 or more daily dose administrations. The observation period should be extended for 10 days following the last dose administered.

REPORTING OF DATA

The following is a check list of considerations used in reporting approximate maximum tolerated dose studies of pesticides on wildlife. Appropriate items are to be noted on control and treated test animals or groups before, after and during the test: Date of Test; Species; Source; Age and how determined; Body weight; Number per Test; Sex Distribution; Feed Description; Feed Consumption; Pesticide Dosing (pesticide formulation, type and description) dosage preparation, diluent concentration, volume administered, mode; Dose Schedule; Signs of toxicity (with day noted); weakness; posture; anorexia; diarrhea; irritability; other; Mortality; Accidental Deaths or Injuries; specifically note other signs indicated in previous mammalian tests; Necropsy Data; Histopathology (if suggested by observations in necropsy); Estimated Maximum Tolerated Dose; single dose administration; repeated dose administration.

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PROTOCOL FOR LABORATORY ACUTE ORAL TOXICITY—BIRDS

The purpose of this test is to determine the single-dose oral LD–50 of a pesticide to wild avian species under laboratory conditions. The information accumulated by completing this test will be used along with

other toxicity information to evaluate the hazards of the chemical to wildlife.

The following protocol is a clarification of the USDA acute oral protocol (Exhibit #1) with some minor changes. The USDA protocol was the only protocol found that was detailed enough to be recommended as acceptable for use by EPA for their pesticide registration guidelines. Other references examined for wild birds are similar but lack specific details.

METHODS AND MATERIALS

Species and History. The test species of choice are pen-reared Bobwhites and Mallards not less than 16 weeks of age. Birds should not have been mated before being placed on test. A history of rearing practices such as photoperiod, medication, type of food, etc., should be included in the report.

All lots of birds should be healthy and uniform and without deformity. All lots should be weighed, then held for a minimum of 14 days prior to the test. If done indoors, temperature and humidity should be controlled, with freedom from drafts and sudden noises which could disturb the test birds. Any lots of birds that suffer an abnormal weight loss or above 10 percent mortality during the holding period should be discarded.

Cages. All birds shall be caged by treatment level groups or subgroups under acceptable animal husbandry practices.

Weight. All birds must be weighed in grams at the beginning of the test, and on days 3, 7, and 14 after oral administration. Also, birds should be weighed at termination of the test if it extends beyond 14 days.

Diet and fasting. Feed and water must be withheld from the control and test birds (Bobwhites and Mallards) for 15 hr prior to oral administration of the chemical. At all other times, birds shall have free access to feed and water. The diet fed must be a standard feed ration known to be adequate for game birds and without antibiotics.

Feed consumption shall be determined weekly for treated and control birds. The feed consumed must be reported as average daily food consumption for each dose level.

Preparation of test material. 1. When a diluent is required, the preferred diluent is distilled water. However, when a test chemical is not water-soluble, other diluents which may be used include table-grade corn oil, propylene glycol, and 1 percent carboxymethylcellulose. Diluents which have known toxic or emetic properties at the level used are to be avoided.

Recommended maximum amount of diluent per dosage is 0.1 to 1 percent of body weight. The total volume for all dosage levels should have a constant volume to body weight factor.

The diluent is to be administered to control birds at the same volume given test birds.

2. When a diluent is not used, the technical material should be accurately weighed directly or microsyringed into gelatin capsules from precisely diluted acetone solutions. The acetone should be completely evaporated off at room temperature before the capsules are used.

Method of administration. The test chemical is placed by oral intubation into the crop or proventriculus.

Number of birds per dose level. The number of test birds per treatment level shall be no less than 5 of each of sex. The number of control birds shall be no less than 5 of each sex. The number of birds per treatment level and the number of controls shall be reported.

Dosage-mortality data. The factor between dosage levels used to determine the acute LD–50 should be based on a geometric or logarithmic scale. There should be a minimum of 5 dosage levels used in calculating the acute oral LD–50.

The determination of an acute oral LD–50 shall follow any acceptable method. Two examples of acceptable methods are Thompson and Weil (1952) and Litchfield and Wilcoxon (1949).

Period of observation. The test animals shall be observed until death or for a minimum of 14 days. The observation period should be longer if signs of toxicity are still evident. The time of all deaths shall be recorded by day. If more than 10 percent of the control birds die during the test period, the test shall be considered invalid.

Recording of signs. All signs of intoxication shall be recorded as they occur.

REPORTING OF DATA

Should include pertinent information on the items listed below. The metric system should be used for units of measure. Dates of test; Species and rearing history; Sources of birds; Age and sex; Body weights; Diet used; Pen dimensions; If pen indoors or outdoors; Weather condition, if outdoors; Temperature and humidity data, if indoors; Feed consumption; Preparation of test material; Method of administration; Amount of test material dosed per bird; Amount of diluent dosed per bird, if used; Number of birds per treatment level; Number of controls employed; Method used for calculating LD–50; Length of observation period; Time of mortalities; Record of all signs of intoxication or if any regurgitation occurs.

The determined LD–50 and the confidence limits shall be reported in the following manner:

Test chemicals and purity or formulation.	Bird species and sex.	LD–50 in milligrams per kilogram (confidence limits).

Additional data. Necropsies could give valuable additional data, but are not necessarily required. When necropsies are conducted, they should include a general inspection of the digestive tract, liver, kidneys, heart and spleen at the time of death and at the termination of the test for both control and test birds. Any gross pathological changes should be reported.

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U.S. Dept. of Agriculture. 1968. Bobwhite Quail Laboratory Acute Oral Toxicity Test Method (Test A–2). Pesticides Regulation Division, Washington, D.C. (Exhibit 1)

EXHIBIT 1

BOBWHITE QUAIL LABORATORY ACUTE ORAL TOXICITY TEST METHOD

Pesticides Regulation Division

Rodenticides Evaluation Staff

Conditions and procedures described below relate to routine measurements of acute oral toxicity or pesticides to bobwhite quail under laboratory conditions. The results of these tests may be used only as a general guide to the toxicity class of a given pesticide. The nature of some pesticides may require that chronic, dermal and inhalation tests be conducted to further evaluate certain characteristics of the chemical. The acute oral toxicity protocol is not a valid method for chronic studies.

Evaluation as to the hazard of a formulation in the environment may require field testing.

METHODS AND MATERIALS

A. *Species.* The test species of choice is pen reared bobwhite quail not less than 16 weeks of age. (However, if bobwhite quail are unavailable, a second choice would be pen reared Coturnix quail not less than 10 weeks of age). Birds should not have mated before being placed on test.

All lots of birds should be healthy and uniform and without deformity. All lots should be weighed, then held indoors 21 days under controlled temperature and humidity prior to using them in a test. Any lots of birds that suffer an abnormal weight loss or 10% mortality during the holding period should be discarded.

The test room should also have controlled temperature and humidity as well as freedom from drafts and sudden noises which will disturb the birds.

B. *Sex.* The sexes of the birds placed on test and on control shall be equally divided. All birds shall be individually caged. The minimum cage size for bobwhite quail shall be no less than 12" x 20" x 10" high, with food and water placed on the outside of the test cage.

C. *Weight.* Weight must be recorded in grams for all birds at the beginning of the test. Three days after dosing, all surviving birds must be weighed again and every three days thereafter to the termination of the test.

D. *Diet and fasting.* All birds shall have free access to feed and water prior to and after dosing. The feed diet must be a standard feed ration known to be adequate for game birds and without antibiotics. Feed and water must be withheld from the control and test birds for 15 hours prior to dosing.

Feed consumption shall be determined daily. The feed consumed must be reported as average daily food consumption for each dose level.

E. *Preparation of test material.* When the test chemical is in liquid form it must be administered in that form. When a diluent is required the preferred one is distilled water. However, when a test chemical is not water soluble, other diluents which may be used are: table grade corn oil, propylene glycol, carboxymethylcellulose, and normal saline. Diluents which have known toxic properties are to be avoided.

The diluent is to be administered to control birds at the same dose level given test birds, but without the test chemicals.

The diluent shall be reported.

F. *Method of administration.* The method of administration of the test chemical may be by encapsulation or by oral injection with a syringe equipped with a flexible catheter.

The method of administration shall be reported.

G. *Number of birds per dose level.* After a dose range has been determined, by range finding techniques, the number of test birds per dose level shall be no less than 10 of each sex.

The number of control birds shall be no less than 10 of each sex.

The number of birds per dose level and the number of controls shall be reported.

The determination of an acute oral LD_{50} shall follow the method outlined by Litchfield and Wilcoxon.*

The determined LD_{50} and the confidence limits shall be reported in the following manner:

Test Chemical & Purity, Bird Species & Sex, LD_{50} in Mg/Kg. (Confidence Limits)

H. *Dose Interval.* After the general toxicity range has been determined, the dose interval used to determine the acute LD_{50} should be based on a geometric or logarithmic scale. The dose range should provide not more than 20% mortality at the low end of the scale, 40–60% mortality at the center of the scale, and not less than 80% mortality at the upper end of the scale.

I. *Period of Observation.* The test animals shall be observed until death or for 21 days after dosing. The time of death shall be recorded by day.

If 20% or more of the control birds die during the test period, the test shall be considered invalid.

J. *Recording of Symptoms.* When symptoms of intoxication occur, they shall be recorded daily.

K. *Necropsies.* A general inspection of the digestive tract, liver, kidneys, and spleen should be made at the time of death or at the end of the test period for both control and test birds. Any gross pathological changes shall be reported.

L. Reporting of the experiment shall include pertinent information on the above listed sections A through K. The mortality curve, plotted on logarithmic-probability graph paper, and a copy of the laboratory worksheet must accompany the final report.

PROTOCOL FOR DETERMINING LETHAL DIETARY CONCENTRATIONS OF CHEMICALS TO BIRDS (5 DAY-DIETARY-LC–50)

The determination of the relative toxicity of economic poisons to birds is valuable in assessing the potential hazard of chemicals to wildlife. Because the route of exposure of chemicals in birds is commonly through ingestion, this test procedure is appropriately useful. This basic method as described by Heath and Stickel (1965) and Heath et al. (1972), is the only published protocol on avian LC–50 determination.

METHODS AND MATERIALS

Test. This method determines a chemical's toxicity to birds expressed as the median lethal concentration (LC–50) of chemical (technical material) in dry diet. The LC–50 being the parts per million (ppm) toxicant in an *ad libitum* diet expected to produce 50 percent mortality among 2 to 3 week old birds in 8 days (5 days on treated diet followed by 3 days on untreated diet).

Species. This protocol is appropriate, with modifications, for most bird species, but it addresses primarily the Mallard and Bobwhite.

Source of strain of birds. Test birds should be pen-reared and phenotypically indistinguishable from wild birds. It is recommended that birds be utilized only from colonies that have had breeding histories maintained for them.

Age and sex. Test birds should be neither less than 10 days nor greater than 15 days of age at the initiation of the test. It should be noted that LC–50 determinations may be affected by the age of the birds at the time of testing.

A random selection of brids without regard to sex should be used because determination of sex is not feasible at this age.

Health of Birds. Only birds in apparent good health should be used. Birds with obvious abnormalities, injuries or sickly in appearance should be excluded.

Pen Facilities. Pen conditions should conform to good husbandry practices. The following appears satisfactory:

Size. Bobwhite (about 10 birds per pen) may be tested in commercial brooder units with individual pens measuring approximately 35 x 100 x 24 cm high. Waterfowl (Mallards) may be tested in pens 70 x 100 x 24 cm high. Floors and external walls can be of wire mesh, while ceilings and common walls may be of galvanized sheeting.

Temperature and Relative Humidity (RH). For Bobwhites and Mallards, temperature should be about 35°C. It can be maintained with thermostatically controlled central heating. The relative humidity should be about 30–80 percent. Adequate ventilation should be maintained.

Lighting. Fluorescent or incandescent lighting may be supplied varying light regimens. It may follow the diurnal variation or be maintained continuously 24 hours per day.

Test Diets. A standard commercial game bird diet or its equivalent should be used. Water should be continuously available.

To prepare test diets, the chemical may be dissolved in table-grade corn oil or other necessary vehicle (i.e. propylene glycol) and mixed thoroughly with dry commercial mash in a ration of 2 parts of solution to 98 parts of feed by weight. An equivalent amount of vehicle should be added to untreated diets. Diets can be mixed by commercial, mechanical food mixers. Only freshly mixed diets should be used.

Number of Concentrations. Generally, each chemical should be administered in 6 dietary concentrations spaced geometrically over a span intended to produce mortality ranging from 10 to 90 percent. It is advisable to perform a range finding study prior to selecting concentrations for testing.

Test birds should be preconditioned to the test facilities for at least one day prior to testing and fed untreated food prior to dosage.

Number of Birds/Concentration. From 6 to 15 birds or more may be used per concentration; 10 birds per concentration seems to be practical and is statistically useful.

Use of Dieldrin Standard. A dieldrin "standard" of 6 concentrations plus 3 pens of controls can be included with any given set of chemicals being tested to identify possible differences in animal sensitivity between experiments. Dieldrin "standard" tests may be optional at given laboratories that have established the "standard" in prior tests for assessing their bird colonies and that are repeatedly conducting LC–50 determinations. It is advisable to annually conduct dieldrin "standard" tests to assure continued uniformity of bird colonies.

Observations on Signs of Intoxication. Throughout the test, all signs of intoxication and abnormal behavior should be noted. Any unusual manifestation of the chemical to the test birds should be recorded.

Food Consumption. Estimates of average food consumption as measured daily per selected pens should be made for birds in each test. Although food spillage hinders this calculation, this datum, along with other information may be useful in the evaluation of the hazards or repellency of a particular chemical.

Statistical Design. A completely randomized design should be used in each experiment with treatments and birds randomly assigned to pens.

Statistical Procedure for Handling Data. The LC–50 values and associated statistics can be derived by methods of probit analysis as described by Finney (1952) and programmed for computer by Daum and Killcreas (1966). The application of the basic probit method by Litchfield and Wilcoxon (1949), Weil (1952), Miller and Tainter (1944) or other appropriate sources may be utilized.

REPORTING OF DATA

The following information and data should be reported: Dates of test; Species; Breeding history of test birds; Source and strain of test birds; Age (days); Sex; Housing conditions (including temperature, RH., lighting); Test diets; Chemical tested (chemical structure); Diluent(s) used; Number of concentrations of chemical used; Number of birds per concentration; Standard used; Signs of intoxication (including unusual responses to chemical by test birds); Food consumption; LC–50 determinations (ppm) with 95 percent confidence limits.

ADDITIONAL DATA (IF COLLECTED)

Analysis of concentrates; Length of observations (if observation period extended); gross pathology.

Additional Considerations—Analysis of Concentrates. It may be desirable to sample and analyze the chemical diet pre-mix concentrate to determine the level of chemical used in treated diets.

Length of Observation. The period after the 5-day exposure of birds to treated diets should be extended as long as test birds exhibit toxic signs or birds die.

Gross Pathology. Pathological examination should be conducted on a sample of birds that die and some survivors to identify gross lesions. These data may be useful for diagnostic purposes related to mortality of birds that might die in the field.

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REPRODUCTION TESTS ON BIRDS

The Environmental Protection Agency may require reproductive studies with some pesticides on the Bobwhite and Mallard, under certain circumstances, for the registration of the pesticide. Results from such tests should reflect possible kinds of effects of any pesticide on all reproductive parameters if

*Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharm. and Exp. Therap.* 96:99–113 (May–August).

possible, including chronic effects. Reproductive parameter measurements should include effects on the adult, egg and hatched young. Pesticides which may require such testing are those which are persistent, are stored or accumulate in plant or animal tissues, or are used under conditions where wild birds may be subjected to repeated or continuous exposure.

Although the reproductive parameters measured for both species are similar, the detailed information required makes it necessary to have a separate protocol on each species. These protocols themselves are not complete but furnish, along with the more specific information in references cited, the backbone of information necessary and desired to properly evaluate the effects of pesticides on the reproduction of the Bobwhite and Mallard.

Most of the bird reproduction data in the literature dates from 1970 and has been on the chicken, Japanese quail, Ring-necked pheasant and certain species of doves and finches, etc. No suitable complete protocol was available from any of the literature for the Bobwhite; more information (but still insufficient) was available for the Mallard, especially in respect to the egg-shell thinning parameter of bird reproduction. Literature references have been written by researchers already familiar with bird tests, so many details were not included in the protocol of such writeup. Therefore, the recent unpublished literature, as well as published literature, was used as a basis, not for writing new test methods, but for preparing a composite of information on the Bobwhite and Mallard which would constitute working protocols on reproduction.

The reproduction test is also useful as a measure of chronic toxicity as well as teratogenicity. It is a logical test to follow the 5-day dietary LC–50 test and to do before field testing on a large scale. Care must be taken with measuring the reproduction parameters since there is considerable variation depending on the care taken in all stages of the test. Since all tests are compared with the control, misleading comparisons on test chemical evaluations may occur if normal variations in test data are not established in control birds for each parameter.

PROTOCOL FOR EVALUATION OF REPRODUCTIVE EFFECTS OF PESTICIDES ON THE BOBWHITE

METHODS AND MATERIALS

Test animals. Pen-reared Bobwhite previously untreated approaching their first breeding season, phenotypically indistinguishable from wild birds, should be used as test animals. All birds should be examined following shipment for possible physical injury that may have been encountered in transit. If deemed necessary, several birds may be randomly selected for pretreatment necropsy at a diagnostic laboratory to assess the state of health upon arrival.

It is recommended that a history of rearing practices be ascertained. This history should include lighting practices during rearing, disease record, drug and any other medication administered and exact age.

If Bobwhite that have completed one or more breeding seasons are to be used as test animals, then they must be obtained prior to the onset of egg production for another breeding season.

Test groups. A minimum of 3 test groups of Bobwhite should be used. One group must serve as a control and 2 groups as treated birds.

By random distribution, 1 male and 2 females per pen, replicated by a minimum of 12 pens, should be used. If 1 male and female per pen are used, additional pens should be added.

Group No.	Dosage level	Number of pens	Birds/pen	
			Male	Female
1—control	0	12	1	2
2—treated	X parts per million.	12	1	2
3—treated	(n)X parts per million.	12	1	2

NOTE.—(n)=some multiple.

Diet preparation. Concentrations of test substances should be used in relationship to residues expected or determined from the proposed registered use patterns for the compounds. The highest nonlethal level may be estimated from data used in calculating the 5-day dietary LC–50.

Test material should be added to table grade corn oil or other appropriate vehicle and premixed with an aliquot of basal diet utilizing a mortar and pestle or mechanical blender. It is recommended that the aliquot of basal diet used for the premix be screened to remove large particles of diet before blending in the corn oil and test material. The final diet should be a uniformly mixed composition consisting of 98 or 99 parts by weight of the basal diet and 2 or 1 parts by weight of corn oil. The basal diet should be a commercial game bird breeder ration or its equivalent and treated with an equivalent amount of vehicle. The premix should be stored frozen until utilized for preparation of the test diet. Test diets should be prepared periodically to prevent deterioration of the diet. Test diets should be analyzed for pesticide concentrations at intervals during the tests.

Testing Phase—Test Environment. The Bobwhite should be housed in breeding pens of adequate size conforming to good husbandry practices.

Since light treatment is extremely important, both during rearing and during the egg laying period, for maximum egg production all Bobwhite should be maintained for the first 8 weeks under a regime of 7 hours of light per day.

The photoperiod should then be increased to 16–17 hours of light per day and either maintained at this level or increased by 15 minutes per week for the following 12 weeks. (The 12-week period may vary depending upon the time required for the onset of egg production.) An illumination intensity of 5 ft-candles at the bird level during the lighting phase of the reproductive study is adequate. Avoid the use of shorter wave length "cool white" fluorescent lights which do not simulate the daylight spectrum.

Temperature and relative humidity control throughout the reproductive test is desirable and should be recorded. Recommended levels approximate 21°C and 55 percent relative humidity. Ventilation is necessary.

Feeding and Husbandry. All birds should receive the appropriate diet *ad libitum* for the duration of the study. Water is to be provided *ad libitum*. The test chemical should be administered not less than 10 weeks before egg laying is expected.

Body weights should be recorded at weeks 0, 2, 4, 6, 8 and at the termination of treatment. During egg laying body weight recording is discouraged because of the adverse effects that handling may have on egg production.

Food consumption should be recorded at least at biweekly intervals throughout the study.

Mortality should be recorded by date and morbidity noted together with clinical signs throughout the test phase. Gross pathology data should be considered for birds that die during the course of the test phase and for some survivors.

Egg Collection, Storage and Incubation. All eggs should be collected daily, marked according to pen from which collected, and stored at 16°C and 65 percent relative humidity. Eggs should be set at weekly intervals for incubation in a commercial incubator. All eggs should be candled on Day 0 for eggshell cracks; on approximately Day 11 to measure fertility and early death of embryos; and on Day 21 to measure embryo survival. Transfer should be made on Day 21 to a separate commercial incubator or hatcher for hatching.

Recommended temperatures and relative humidity during incubation are approximately 37.5°C and 60 percent relative humidity. Recommended temperatures and relative humidity during hatching phase are approximately 37°C and 70 percent, respectively.

Chick Observation. On Day 24 of incubation the hatched chicks should be removed, hatchability recorded, and chicks housed according to the appropriate parental grouping and maintained on control diet for 14 days (extend if appreciable late mortality occurs). The diet should be a commercial Bobwhite starter diet or its equivalent.

Eggshell Thickness. All eggs from 1-day's collection at biweekly intervals should be measured for eggshell thickness. Preferably, eggs laid in the first part of weeks 1, 3, 5, 7, and 9 of the egg-laying period should be used for eggshell measurements. An accepted procedure is to crack open the eggs at the widest portion (girth or waist), wash out all egg contents, air dry the shells at least 48 hours, and then measure the thickness of the dried shell plus the membranes at 3 or 4 points around the girth using a micrometer calibrated to 0.01 mm units.

Analysis. Experimental groups should be compared to the control group by analysis of variance. Sample units are the individual pens within each experimental group. Parameter analyses should include: body weight, food consumption, eggs laid, eggs cracked, eggs set, fertility, live 3-week embryos, hatchability, normal chicks, 14-day-old survivors per number of eggs hatched and per hen, and eggshell thickness.

Definitions and Typical Observed Values. The values reported here represent those observed from a few facilities under their conditions and are not necessarily representative of those from all facilities.

1. Eggs laid

Definition. The total egg production during a breeding season (which is approximately 10 weeks).

Normal Values. 28 to 38 eggs per hen per season.

2. Eggs cracked

Definition. Eggs determined to have cracked shells when inspected with a candling lamp; fine cracks cannot be detected without utilizing a candling lamp and if undetected will bias data by adversely affecting embryo development.

Normal Values. 0.6 percent to 2.0 percent.

3. Eggs set

Definition. All eggs placed under incubation, i.e., total eggs laid minus cracked eggs and those selected for eggshell thickness analysis. The only value in presenting this parameter is to establish a base of reference for viable embryos.

4. Viable embryos

Definition. Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the egg 6 to 14 days after incubation has begun. It is difficult to distinguish between the

absence of fertilization and an early embryonic death. This distinction can be made by breaking out eggs that appear infertile and examine further. This is especially important in the event a test compound induces early embryo mortality.

Normal Values. 75 percent to 90 percent.

5. Live 3-week embryo

Definition. These are embryos that are developing normally after 3 weeks of incubation. This is determined by candling the egg.

Normal Values. 97 percent to 99 percent.

6. Hatchability

Definition. Embryos that mature, pip the shell, and liberate themselves from their eggs on Day 23 or 24 of incubation (computed from the number of fertile eggs).

Normal Values. 50 percent to 90 percent.

7. 14-day-old survivors

Definition. Birds that survive for 2 weeks following hatch.

Normal values. 75 percent to 90 percent.

8. Eggshell thickness

Definition. The thickness of the shell and the membrane of the egg at the girth after having been opened and washed, and dried for at least 48 hours at room temperature.

Normal values. 0.20 mm to 0.22 mm.

Additional Data. If the test substance is toxic (reduced reproduction evident) then a withdrawal study period may be added to the test phase. The withdrawal period need not exceed 8 weeks. Continued observations can be made on egg production, fertility, hatchability and hatchling survival.

REPORTING OF DATA

Basic data supporting the above analyses should be tabulated. All abnormal behavior, morphological, physiological or other parameters observed should be included. Interpretation of test conditions or results known to be unusual or anomalous should be included if possible. All items in the following list should be considered when reporting results.

Appropriate items are to be noted on untreated and treated test groups before, after and during the test: Dates of test; Species; Strain; Source; Age; Body weight; Number per test (include sex ratio); Individual identification of birds; Diet; Storage; Feed consumption (grains per day); Observations on palatability or repellency.

Housing conditions of test birds; Include space allocations for mating, nesting; Include protection from weather, injuries; Lighting program including hours per day, wattage or footcandles at bird level; Diagram of test layout; Temperature; Water supply; Morbidity; Mortality; Accidental deaths or injuries; Pretest and test history of medical and chemical administration; Observable clinical signs; Clinical tests; Post mortem autopsy.

Egg data; Shell Thickness; Cracked Eggs; Eggs Laid (number eggs per bird per day and per season); Hatching egg storage data; Temperature; Humidity; Days; Incubation data; Eggs set; Temperature; Humidity; Turning Frequency; Fertility (viable embryos); Live 3-week embryos; Hatchability; Pipped; Dead embryos; Post-hatching (juvenile birds); Cripples; Morbidity; Mortality (14-day-old survivors); Weight; Clinical signs.

Application of pesticide (judge toxicant levels based on field application calculations); Formulation (in corn oil); Method (dietary-acute, capsule, water?); Analytical or bioassay method; Pesticide dosage in food (ppm); Pesticide dosage in bird (mg/kg/day); Stability of pesticide in diet.

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PROTOCOL FOR EVALUATION OF REPRODUCTIVE EFFECTS OF PESTICIDES ON THE MALLARD

METHODS AND MATERIALS

Test Animals. Pen-reared Mallards previously untreated approaching their first breeding season, phenotypically indistinguishable from wild birds, should be used as test animals. All birds should be examined following shipment for possible physical injury that may have been encountered in transit. If deemed necessary, several birds may be randomly selected for pre-treatment necropsy at a diagnostic laboratory to assess the state of health upon arrival.

It is recommended that a history of rearing practices be ascertained. This history should include lighting practices during rearing, disease record, drug and any other medication administered and exact age.

If Mallards that have completed one or more breeding seasons are to be used as test animals, then they must be obtained prior to the onset of egg production for another breeding season.

Test Groups. A minimum of 3 test groups of Mallards should be used. One group must serve as a control and 2 groups as treated birds.

By random distribution a minimum of 2 males and 5 females per pen, replicated by 4 or more pens, should be used. If individual pairs (1 male and 1 female) are to be used per pen, a minimum of 10 pens should be employed. The tables below illustrate these 2 options:

Group No.	Dosage level	Minimum number of pens	Birds/pen	
			Male	Female
1—control	0	4	2	5
2—treated	X parts per million.	4	2	5
3—treated	(n)X parts per million.	4	2	5

NOTE.—(n)=Some multiple.

Group No.	Dosage level	Minimum number of pens	Birds/pen	
			Male	Female
1—control	0	10	1	1
2—treated	X parts per million.	10	1	1
3—treated	(n)X parts per million.	10	1	1

NOTE.—(n)=Some multiple.

Diet Preparation. Concentration of test substances should be used in relationship to residues expected or determined from the proposed registered use patterns for the compound. The highest nonlethal level may be estimated from data used in calculating the 5-day LC–50 level.

Test material should be added to table grade corn oil or other appropriate vehicle and premixed with an aliquot of basal diet utilizing a mortar and pestle or mechanical blender. It is recommended that the aliquot of basal diet used for the premix be screened to remove large particles of diet before blending in the corn oil and test material. The final diet should be a uniformly mixed composition consisting of 98 or 99 parts by weight of the basal diet and 2 or 1 parts by weight of corn oil. The basal diet should be a commercial game bird breeder ration or its equivalent and treated with an equivalent amount of vehicle. The premix should be stored frozen until utilized for preparation of the test diet. Test diets should be prepared periodically to prevent deterioration of the diet.

Test diets should be analyzed for pesticide concentration at intervals during the tests.

Test Environment. The Mallards should be housed in breeding pens of adequate size conforming to good husbandry practices. Preferably the pens should be screen-bottomed or kept clean of spilled treated food and excrement.

Since light treatment is extremely important, both during rearing and during the egg laying period, for maximum egg production, all Mallards should be maintained for the first 8 weeks under a regime of 7 hours of light per day.

The photoperiod should then be increased to 16–17 hours of light per day and either maintained at this level or increased by 15 min per week for the following 12 weeks. (The 12-week period may vary depending upon the time required for the onset of egg production.) An illumination intensity of 5 footcandles at the bird level during the lighting phase of the reproductive study is adequate. Avoid the use of shorter wave length "cool white" fluorescent lights which do not simulate the daylight spectrum.

Temperature and relative humidity control throughout the reproductive test is desirable and should be recorded. Recommended levels approximate 21°C and 55 percent relative humidity. Ventilation indoors is necessary.

Feeding and Husbandry—All birds should receive the appropriate diet *ad libitum* for the duration of the study. Water is to be provided *ad libitum*. The test chemical should be administered not less than 10 weeks before egg laying is expected.

Body weights should be recorded at weeks 0, 2, 4, 6, 8 and at the termination of treatment. During egg laying, body weight recording is discouraged because of the adverse effects that handling may have on egg production.

Food consumption should be recorded at least at biweekly intervals throughout the study.

Mortality should be recorded by date and morbidity noted together with clinical signs throughout the test phase. Gross pathology data should be considered for birds that die during the course of the test phase and for some survivors.

Egg Collection, Storage and Incubation—All eggs should be collected daily, marked according to pen from which collected, and stored at 16° C and 65 percent relative humidity. Eggs should be set at weekly intervals for incubation in a commercial incubator. All eggs should be candled on Day 0 for egg-

shell cracks; by Day 14 of incubation to measure fertility and early death of embryos, and on Day 21 to measure embryo survival. Transfer should be made on Day 23 to a commercial incubator for hatching.

Recommended temperatures and relative humidity during incubation are 37.5° C and 60 percent respectively. Recommended temperatures and relative humidity during hatching phase are 39° C and 70 percent respectively.

Duckling Observations. On Day 27 of incubation the hatched Mallard ducklings should be removed, hatchability recorded and ducklings housed according to the appropriate parental grouping and maintained on control diet for 14 days (extended if appreciable late mortality occurs). The diet should be a commercial Mallard starter diet or its equivalent.

Eggshell Thickness—All eggs from 1-day's collection at biweekly intervals should be measured for eggshell thickness. Preferably, eggs laid in the first part of weeks 1,3,5,7 and 9 of the egg-laying period should be used for eggshell measurements. An accepted procedure is to crack open the eggs at the widest portion (girth or waist), wash out all egg contents, air dry the shells at least 48 hours, and then measure the thickness of the dried shell plus the membranes at 3 or 4 points around the girth using a micrometer calibrated to 0.01 mm units.

Analysis—Experimental groups should be compared to the control group by analysis of variance. Sample units are the individual pens within each experimental group. Parameter analyses should include: body weight, food consumption, eggs laid, eggs cracked, eggs set, fertility, live 3-week embryos, hatchability, normal ducklings, 14-day-old survivors per number of eggs hatched and per hen; and eggshell thickness.

Definitions and Typical Observed Values. The values reported here represent those observed from a few facilities under their conditions and are not necessarily representative of those from all facilities.

1. Eggs laid

Definition. The total egg production during a breeding season (which is approximately 10 weeks).

Normal Values. 28 to 38 eggs per hen per season.

2. Eggs cracked

Definition. Eggs determined to have cracked shells when inspected with a candling lamp; fine cracks cannot be detected without utilizing a candling lamp and if undetected will bias data by adversely affecting embryo development.

Normal Values. 0.6 percent to 6 percent.

3. Eggs set

Definition. All eggs placed under incubation, i.e., total eggs laid minus cracked eggs and those selected for eggshell thickness analysis. The only value in presenting this parameter is to establish a base of reference for viable embryos.

4. Viable embryos (fertility)

Definition. Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the egg 6 to 14 days after incubation has begun. It is difficult to distinguish between the absence of fertilization and an early embryonic death. This distinction can be made by breaking out eggs that appear infertile and examine further. This is especially important in the event a test compound induces early embryo mortality.

Normal Values. 85 percent to 98 percent.

5. Live 3-week embryo

Definition. These are embryos that are developing normally after 3 weeks of incubation. This is determined by candling the egg.

Normal Values. 97 percent to 99 percent.

6. Hatchability

Definition. Embryos that mature, pip the shell, and liberate themselves from their eggs on Day 25 to 27 of incubation (computed from the number of fertile eggs).

Normal Values. 50 percent to 90 percent.

7. 14-day-old survivors

Definition. Birds that survive for 2 weeks following hatch.

Normal Values. 94 percent to 99 percent.

8. Eggshell thickness

Definition. The thickness of the shell and the membrane of the egg at the girth after having been opened and washed, and dried for at least 48 hours at room temperature.

Normal Values. 0.31 mm to 0.38 mm.

REPORTING OF DATA

Basic data supporting the above analyses should be tabulated. All abnormal behavior, morphological, physiological, or other parameters observed should be included. Interpretation of test conditions or results known to be unusual or anomalous should be included if possible. All items should be considered when reporting results.

Appropriate items are to be noted on untreated and treated test groups before, after and during the test: Dates of test; Species; Strain; Sources; Age; Body weight; Number per test (include sex ratio).

Individual identification of birds; Diet; Storage; Feed consumption (grams per day); Observations on palatability or repellency; Housing conditions of test birds; Include space allocations for mating, nesting; Include protection from weather, injuries; Lighting program including hours per day, wattage of foptcandles at bird level; Diagram of test layout; Temperature; Water supply; Morbidity; Mortality; Accidental deaths or injuries; Pretest and test history of medical and chemical administration; Observable clinical signs; Clinical tests; Post mortem autopsy.

Egg data; Shell thickness; Cracked eggs; Eggs laid (number eggs per bird per day and per season); Hatching egg storage data; Temperature; Humidity; Days; Incubation data; Eggs set; Temperature; Humidity; Turning frequency; Fertility (viable embryos); Live 3-week embryos; Hatchability; Pipped; Dead embryos; Post-hatching (juvenile birds); Cripples; Morbidity; Mortality (14-day-old survivors); Weight; Clinical signs.

Application of pesticide (Judge toxicant levels based on field application calculations); Formulation (in corn oil); Method (dietary-acute, capsule, water?); Analytical or bioassay method; Pesticide dosage in food (ppm); Pesticide dosage in bird (mg/kg/day); Stability of pesticide in diet.

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EXHIBIT 1

A GUIDELINE FOR EVALUATION OF REPRODUCTIVE EFFECTS OF ECONOMIC POISONS ON AVIAN SPECIES

There is strong evidence to indicate that a chemical can be innocuous in terms of direct mortality and yet impair reproductive success. Reproduction studies to evaluate certain parameters of reproductive effects similar to the following may be required.

METHODS AND MATERIALS

1. *Species.* Mallard, as a representative aquatic bird, and either bobwhite quail or ring-necked pheasant as a representative upland game bird. The coturnix quail is not acceptable for reproduction studies.

2. *Age and Sex.* Young adult males and females approaching their first breeding season.

3. *Diet.* Any standard commercial game bird breeder mash is adequate. Matchlings are immediately placed on a clean diet.

4. *Test Plan.* The pen is the basic testing unit. Each pen shall contain 5 females and 2 males except when ring-necked pheasant is the test species. In such case only one male per pen is used.

Birds shall be housed in suitable cages or brooders with water and food provided *ad libitum*. The test chemical (Technical grade) shall be dissolved in edible-grade corn oil prior to incorporation in the ration. If the test material is incompatible with, or insufficiently soluble in corn oil, propylene glycol may be used as the solvent. Concentration of test chemical in the solution shall be adjusted so that the addition of 1 part (by

weight) of this solution to 99 parts of the standard commercial gamebird ration will result in the desired concentration (ppm) of test chemical in the finished diet. Ration for control birds is mixed with solvent (free of chemical) in the same ratio.

A completely randomized design is used throughout. The 5 females and male(s) allotted to a pen represent a pen set. Four sets of controls (chemical free diet) are determined concurrently. Two treatment levels are preferred. Each treatment level is replicated in a minimum of four pens.

Treatment	Number of pens (minimum)	Male-female/pen	Number of birds (minimum)
Control	4	2 to 5	28
Test chemical (level 1).	4	...do	28
Test chemical (level 2).	4	...do	28

5. *Treatment Levels*—When estimating the highest level (level 1) the birds can sustain for the test period some knowledge of the toxicity of the chemical is necessary. The highest non-lethal level (level 1) is estimated, using the data obtained from the LC_{50} determination. A second level 60 percent lower than level 1 is selected. This "safety level" can prove invaluable in the event the higher level proves to be lethal prior to the reproductive period. If, however, a chemical is expected to produce no appreciable mortality at the highest recommended field application rate, it is tested at only 1 treatment level estimated to be two times the highest level birds are expected to encounter in the field.

6. *Data Collection*—The test is started at least two months, preferably three, before the beginning of the first laying season and continued through the reproductive cycle. Eggs are collected daily and stored for two weeks under controlled conditions (16° C and 55 percent relative humidity). Examine eggs and discard those with cracked shells. Begin incubation at two-week intervals. Candle after two and three weeks of incubation to measure embryo survival. Hatchlings are immediately placed on clean diet and observed for 14 days. Record the following data for each pen for every two-week set of eggs and determine the parameters of reproductive success listed below:

(a) Total eggs laid.
(b) Eggs laid per hen per two week period.
(c) Eggs cracked (percent).
(d) Eggs embryonated of eggs set (percent).
(e) Embryos alive at three weeks.
(f) Normal hatchlings.
(g) Chick survival to 14 days (percent).
(h) Fourteen-day chicks per hen.

7. *Analysis*. Analyze measurements by analysis of variance (Cochran, 1943). Pen statistics are weighted according to the number of eggs of hatchlings on which they are based. Apply angular transformations to percentages for analysis and separate means by methods of Duncan (1955) and Kramer (1956). A complete description is required if a different analysis technique is used.

8. *Autopsy*. Autopsy data shall be reported on a representative sample of birds from each treatment group.

9. *Observations*. Birds are observed throughout the study and any abnormal behavior is recorded. Record weights of birds at start and end of test. Estimate as accurately as possible food consumption per bird.

10. *Shell Thickness*. At least 10 percent of eggs from each two-week set shall be reserved for shell thickness measurements. Eggs for shell measurements are selected prior to the incubation period. Shell measurements should be made in accordance with procedure described by Longcore et al. (1971). Store contents of eggs used for shell thickness measurements in glass jars and retain for possible residue analysis. It is not anticipated that egg residue analysis will be required on every chemical, however, when a high percentage of embryo failure occurs and/or significant mortality in 14 day hatchlings, residue analysis will be indicated.

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PROTOCOLS FOR LABORATORY ACUTE DERMAL TOXICITY TEST

The following methods can be used to assess dermal hazards of pesticides to wild avian species if it is required by the Environmental Protection Agency.

Protocol #1 is a method that has been used by Schafer *et al.* (1973) to determine the acute dermal LD–50's of pesticides on some wild and domestic birds. This method can be used successfully to determine dermal toxicity of pesticides for most wild and domestic birds that can be conveniently held in captivity.

Protocol #2 was specifically designed for use on Mallards but could be used without any problems on most waterfowl that can be held successfully in captivity (DWRC Annual Reports—manuscripts in preparation).

Both of these methods as published are very general as to how to conduct a dermal LD–50 except for the part on how the birds were dermally treated. Therefore, the majority of the protocol for these methods is a repeat of the acute oral recommended protocol except for the parts that pertain to actual treatment of the birds (See Protocol entitled: Protocol for Laboratory Acute Toxicity).

METHODS #1 AND MATERIALS

Species and history. Almost all species of birds that can be successfully held in captivity can be used with this method.

A history, as complete as possible, of rearing practices such as photoperiod, medication, type of food, etc., should be included in the report.

All lots of birds should be healthy and uniform and without deformity. All lots should be weighed, then held for a minimum of 14 days. If done indoors, temperature and humidity should be controlled. Any lots of birds that suffer an abnormal weight loss or above 10 percent mortality during the holding period should be discarded.

The test room should also have controlled temperature and humidity as well as freedom from drafts and sudden noises which could disturb the test birds.

Cages. All birds shall be individually caged, to meet acceptable animal husbandry practices.

Weight. All birds must be weighed, in grams, at the beginning of the test, and on days 3 and 14 after treatment. Also, birds should be weighed at termination of the test if it extends beyond 14 days.

Diet and fasting. All birds shall have free access to feed and water throughout the test. The diet fed must be a standard feed ration known to be adequate for game birds and without antibiotics.

Feed consumption shall be determined weekly for treated and control birds. The feed consumed must be reported as average daily food consumption for each treatment level.

Administration of test material. Acetone solutions should be prepared so that the total amount administered (in μl) equals 2 times the bird's weight (in g). If the pesticide is not soluble in acetone, any nonhazardous volatile solvent is acceptable. Acetone solutions should be applied with a syringe having a 21-ga, 2.5 cm needle whose tip has been squared and polished to avoid abrading the skin. The solution should be slowly applied to an area 1 cm^2 in the sparsely feathered skin area covering the pectoralis muscles under one wing joint. The acetone should be evaporated by a current of air.

Number of Birds per Dose Level. The number of test birds per treatment level shall be no less than 4 of each sex. The number of control birds shall be no less than 4 of each sex.

Dosage-mortality data. The factor between dosage levels used to determine the acute LD–50 should be based on a geometric or logarithmic scale. There should be a minimum of 4 dosage levels used in calculating the acute dermal LD–50.

The determination of an acute dermal LD–50 shall follow any acceptable method. Two examples of acceptable methods are Thompson and Weil (1952) and Litchfield and Wilcoxon (1949).

Period of observation. The test animals shall be observed until death or for a minimum of 14 days. The observation period should be longer if signs and toxicity are still evident. The time of all deaths shall be recorded by day. If 10 percent or more of the control birds die during the test period, the test shall be considered invalid.

Recording of signs. All signs of intoxication shall be recorded as they occur. Also, the area treated should be checked to determine if any dermal irritation occurs.

REPORTING OF DATA

Should include pertinent information on the items listed below. The metric system should be used for units of measure. Dates of test; Species and rearing history; Source of birds; Age and sex; Body weights; Diet used; Pen dimensions; If pen indoors or outdoors; Controlled temperature and humidity if indoors; Weather condition, if outdoors; Feed consumption; Preparation of test material; Method of administration.

Amount of test material dermally applied per bird; Amount of diluent used per treated bird; Number of birds per treatment level; Number of controls employed; Method used for calculating LD–50; Length of observation period; Time of mortalities; Record of all signs of intoxication.

The determined LD–50 and the confidence limits shall be reported in the following manner: Test Chemicals and Purity or Formulation; Bird Species and Sex; LD–50 in mg/kg (Confidence Limits).

Additional Data. Necropsies could give valuable additional data, but are not necessarily required. When necropsies are conducted, they should include a general inspection of the digestive tract, liver, kidneys, heart and spleen at the time of death and at the termination of the test for both control and test birds. Any gross pathological changes should be reported if necropsies are performed.

References

Litchfield, L. J. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect expirements. *J. Pharmacol. Exp. Theo.* 96(2): 99–133.

Schafer, E. W., R. B. Brunton, N. F. Lockyer, and J. W. DeGrazio. 1973. Comparative toxicity of seventeen pesticides to the quelea, house sparrow, and red-winged blackbird. *Toxicol. Appl. Pharmacol.* 26: 154–157 (See page 155).

Thompson, W. R. and C. S. Weil. 1952. On the construction of tables for moving average interpolation *Biometrics* 8: 51–54.

METHOD NO. 2 AND MATERIALS

Species and history. This method can be used on all species of waterfowl that can be successfully held in captivity.

A history, as complete as possible, of rearing practices such as photoperiod, medication, type of food, etc., should be included in the report.

All lots of birds should be healthy and uniform and without deformity. All lots should be weighed, then held for a minimum of 14 days. If done indoors, temperature and humidity should be controlled. Any lots of birds that suffer an abnormal weight loss or above 10 percent mortality during the holding period should be discarded.

The test room should also have controlled temperature and humidity as well as freedom from drafts and sudden noises which could disturb the test birds.

Cages. All birds shall be individually caged, to meet acceptable animal husbandry practices.

Weight. All birds must be weighed, in grams, at the beginning of the test, and on days 3 and 14 after treatment. Also, birds should be weighed at termination of the test if it extends beyond 14 days.

Diet and fasting. All birds shall have free access to feed and water throughout the test. The diet fed must be a standard feed ration known to be adequate for game birds and without antibiotics.

Feed consumption shall be determined weekly for treated and control birds. The feed consumed must be reported as average daily food consumption for each treatment level.

Administration of test material. The crura of the birds should be shaved with an elastic animal clipper to facilitate taping of the plastic bags with adhesive tape. The pesticide should be dissolved in table-grade corn oil. If it is insoluble in corn oil, then either propylene glycol or distilled water can be used as the diluent. Appropriate amounts of the pesticide should be dissolved in the diluent so that approximately 0.6 ml of the solution is applied to each foot. The area covered on each foot should include the tarso metatarsus, phralanges, and the webbing. The feet should then be covered with a 4 in. x 6 in. plastic bag 0.010 in. thickness, which extends midway up the crus. This exposure should be continued for 24 hr, at which time the bags should be removed and the feet washed with a mild soap and warm water.

Number of birds per dose level. The number of test birds per treatment level shall be no less than 4 of each sex. The number of control birds shall be no less than 4 of each sex.

Dosage-mortality data. The factor between dosage levels used to determine the acute LD–50 should be based on a geometric or logarithmic scale. There should be a minimum of 4 dosage levels used in calculating the acute dermal LD–50.

The determination of an acute dermal LD–50 shall follow any acceptable method. Two examples of acceptable methods are Thompson and Weil (1952) and Litchfield and Wilcoxon (1949).

Period of observation. The test animals shall be observed until death or for a minimum of 14 days. The observation period should be longer if signs of toxicity are still evident. The time of all deaths shall be recorded by day. If 10 percent or more of the control birds die during the test period, the test shall be considered invalid.

Recording of signs. All signs of intoxication shall be recorded as they occur. Also, the area treated should be checked to determine if any dermal irritation occurs.

REPORTING OF DATA

Should include pertinent information on the items listed below. The metric system should be used for units of measure. Dates of test; Species and rearing history; Source of birds; Age and sex; Body weights; Diet used; Pen dimensions; If pen indoors or outdoors; Controlled temperature and relative humidity, if indoors; Weather condition, if outdoors; Feed consumption; Preparation of test material; Method of administration; Amount of test material dermally applied per bird; Amount of diluent used per treated bird; Number of birds per treatment level; Number of controls employed; Method used for calculating LD–50; Length of observation period; Time of mortalities; Record of all signs of intoxication.

The determined LD–50 and the confidence limits shall be reported in the following manner:

Test chemicals and purity or formulation.	Bird species and sex.	LD–50 in milligrams per kilogram (confidence limits).

Additional Data. Necropsies could give valuable additional data, but are not necessarily required. When necropsies are conducted, they should include a general inspection of the digestive tract, liver, kidneys, heart and spleen at the time of death and at the termination of the test for both control and test birds. Any gross pathological changes should be reported if necropsies are performed.

References

Litchfield, L. J. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Theo.* 96(2):99–133.

Thompson, W. R. and C. S. Weil. 1952. On the construction of tables for moving average interpolation. *Biometrics* 8:51–54.

Exhibit 1—Denver Wildlife Research Center Annual Report (a copy presented on next pg).

EXHIBIT 1

PERCUTANEOUS: MALLARDS

Two groups of four hens each were employed. They were 20–23 weeks old and weighed 1.188 (0.919–1.404) kg. The crura of the hens were shaved to facilitate taping of the plastic bags. The chemical was dissolved in water which was then applied in the appropriate amounts to both feet of each bird. Approximately 0.6 ml of solution was applied to each foot. The area covered included the tarso-metatarsus, phalanges, and webbing. The feet were then covered with a plastic bag which extended midway up the crus. This exposure was continued for 24 hours, at which time the bags were removed and the feet washed with "Ivory" soap and warm water. Survivors were observed for 14 days before sacrifice.

PROTOCOL FOR SMALL PEN SIMULATED FIELD TEST TO EVALUATE PESTICIDE HAZARDS TO BIRDS

This protocol has been developed as an initial simulated field test for Bobwhite. With modifications, other species could be tested. This method exposes the test animals to field applications of the chemical. Exposure will not necessarily be similar to that of free-ranging animals.

METHODS AND MATERIALS

Birds. Test subjects shall be obtained from pen-reared stock. All birds shall be maintained in outdoor pens, in the general area where the field test is to be conducted, for at least 2 weeks prior to being placed on test.

Each test shall involve not less than 6 pairs of birds per control group and not less than 6 pairs of birds per test group, with 1 pair of birds per pen. It is recommended that at least 12 additional birds be procured and held in outdoor pens for replacement purposes.

Pens. Each pen shall contain approximately 20 sq ft (4 ft x 5 ft x 12 in. or 3 ft x 7 ft x 12 in., inside measurement), and is to consist of a 2 in. x 2 in. wooden frame covered on the inside with ½ in. mesh hardware cloth. To avoid possible contamination, scrub wire and replace frames if pens have been used for previous testing. Use of aluminum tubing for framing will make cleaning of pens easier. Pen height may be increased from 12 in. to a height that will accommodate vegetative growth through the test period.

Do not cover pen bottom, stake pen securely to the group to minimize predation. Move pens daily, or as required to maintain adequate vegetative cover. Pens should have an opening through which birds can be removed and added. Each pen is to contain a poultry waterer (preferably a 1-qt chick fount) and a small box (open on 1 side, 12 in. x 12 in. x 8 in. high), to serve as a shelter for the birds.

Test conditions. Conditions for evaluation of each pesticidal formulation should approximate those to be encountered in the routine use of the product. Evaluation of a cotton insecticide should be made in a cotton field, and the timing, rate, number, and manner of applications should be identical with those used for control of cotton insects. Similarly, products for control of grass or alfalfa insects should be tested in hay fields, pastures, or meadows. Pens of "control" birds are to be placed in similar habitat not subject to application of the pesticide, with subsequent handling (feeding, watering and observation) the same as for test birds. Clearly mark all pens and all birds to facilitate accuracy in reporting.

Care should be taken at all times to avoid possible contamination through drift from adjacent areas or from improper cleaning of equipment.

Test procedures. Pens and shelters are to be placed in position and birds (1 male and 1 female per pen) introduced prior to application of the pesticide. Also preceding the pesticide application, place filled waterers and approximately 3 oz of cracked corn, wheat or other grain (scattered on the ground) in ½ of the total test and control pens used. The remaining test and control pens are to be left without feed and water for 12 hrs after the pesticide application, at which time feed and water is to be introduced to these pens, as above. Sufficient food and water is to be available to the birds at all times, other than during the indicated 12-hr period.

Follow all safety precautions, as specified on the product label, when entering the treated field.

If either member of the pair dies, the survivor is to be removed, placed in an individual holding pen, and a fresh pair placed in the pen. The survivor should be observed until death or for 14 days. Sacrifice survivors, including "control" group and birds held for replacement, at termination of the experiment.

Duration of test. For pesticides which are to be applied once per season, tests are to be continued for not less than 14 days. For pesticides which are to be applied more than once per season, tests are to be continued for 14 days after the final application, with movement of pens immediately prior to each application.

Reporting of data. Appropriate items to be considered are as follows: Location; Dates; Weather Data; Species; Source; Age; Medical and Chemical Administration History; Body Weight Changes; Individual Identification; Chemical Formulation; Rate of Application; Manner of Application; Vegetative Cover; Residue Analysis; Pen Description; Pen Placement; Diet; Food and Water Supply Schedule; Feed Consumption; Visual Signs of Intoxication; Clinical Measurements for Intoxication; Accidental Deaths or Injuries; Replacement Schedule; Gross Pathological or Histological Examinations; Statistical Methods.

Protocol for Large Pen Simulated Field Studies

For assessing the hazards of pesticides to Bobwhites and Ring-necked pheasants, large cages enclosing portions of habitat of 0.005 ha (500 ft²) or more may be utilized. With modifications, other species can also be tested by this method. Tests utilizing large pens may be conducted for pesticides to be applied on cropland, rangeland, or wildlands or for other outdoor applications such as roadsides, rights-of-way, "waste areas," or other known wildlife habitats. This type of test is not a substitute for full-scale field studies. Pesticides tested in small pens and/or in the large pens may not show effects at as low levels as in actual field operations and thus tend to underestimate the hazard to birds. This is primarily due to two reasons (1) bird exposure to pesticides differs between pen situations and a free environment where they can feed heavily on dying invertebrates (and drink contaminated water etc.) and (2) small, active, insectivorous and omnivorous birds are often more vulnerable to pesticide poisoning than Bobwhites or Ring-necked pheasants, the species used in the tests.

The objective of simulated field tests is to determine pesticide effects on birds under seminatural conditions and to assess the degree of hazard presented by the formulations and application rates of pesticides being considered for registration. Simulated tests can be conducted at less cost and with more control of important parameters and variables than full-scale field studies.

Many pesticide-wildlife studies utilizing penned birds or mammals have been conducted by conservation agencies, industry, and universities. However, most have been specific, one-time studies that apply to a particular field situation. An exhaustive review of these would not add much to development of a general protocol. The Michigan (Black and Zorb 1965, Heezen 1978, Zorb 1968) semifield method, which is the model for this protocol, was developed by a cooperative effort of several agencies and was used to evaluate a number of pesticides over a period of years.

Method and Materials

Pens. Wire-covered pens should be constructed covering a minimum ground area of 0.005 ha (500 ft²) per pen. Suitable pen dimensions might be 3.1 or 3.7 m by 15.2 or 22.9 m (10 ft or 12 ft by 50 ft or 75 ft), with the top cover at a height of about 2.0 m (6.5 ft). Other dimensions covering 0.005 ha (500 ft²) or more per pen may be used. Metal flashing should be placed around all pens to a height of 45.7 cm (18 in.) above ground and to a depth of 15.2 cm (6 in.) below the ground surface.

The number of pens needed to test one chemical is 24 to 36. This would provide 6–9 untreated (control) pens and 6–9 pens for each of 3 treatment levels (the proposed treatment rate and 2 multiples of that rate such as 3 or 5 x and 5 or 10 x). An independent water supply and a small shelter should be furnished in each pen.

Before pens are planned and constructed, the designer and builder should consult wildlife agencies and successful game farms to learn practical considerations such as prevention of parasites and disease, soil drainage requirements, support of top cover to prevent collapse under the weight of snow, types of watering equipment, etc.

Birds. Pens may be stocked with 1 mated pair of Bobwhites per pen. One-year-old birds of known history, not previously exposed to pesticides, shall be placed in the pens at least 2 weeks prior to the pesticide applications. A supply of replacement Bobwhites should be maintained in outdoor pens near the test pens. If either member of a test pair dies, the survivor should be removed and held for observation and a fresh pair placed in the test pen. For practical purposes, a limit should be set on the period of time and number of replacements used.

When pheasants are utilized in this protocol, the pens should be constructed so that removal of one cross-fence would double the pen size, for example, two 3.7 x 22.9 m (12 ft x 75 ft) pens could be converted to one 3.7 x 45.7 m (12 ft x 150 ft) pen. If pheasants are used as the test species, the stocking rate should be 1 male and 5 females per larger pen.

Test conditions and procedures. It would be desirable to use movable pens that can be set up over the crop or vegetation on which the pesticide would be applied, if registered. If permanent (nonportable) pens are used, then the soil should be suitable for growing the pertinent crop or vegetation. The pesticide should be handsprayed with precision at the same rate, timing, number of applications, and formulation as the proposed registration. Replicate pens should also be treated at two multiples (such as 3 or 5 x and 5 or 10 x) of the petition rate. Spraying should be done under minimum wind conditions and with protective shielding to prevent contamination of adjacent sprayed pens and/or control pens. For statistical purposes, it is best to randomize the test pens, but because of the drift problem, it may be better to first stratify the treatment pen locations.

In addition to applying the pesticide in the pens at the proposed rate and 2 multiples of that rate, feed and water should also be treated at the 3 rates. The food treatment rates can be based on results of residue studies required for other purposes in the registration procedure. Treated food (toxicants should be carried in a table grade corn oil, not in dry form or in a repellent solvent) should be prepared within 1 day of the time the pen environments are sprayed. Treated food should be supplied daily or every other day to the test birds in feeders protected from the weather. Food and water treatments should be made with procedures and rates that are consistent with the characteristic of the chemical and the usage being tested. Another desirable phase of the test would be to provide treated animal foods such as grasshoppers or other invertebrates (earthworms, etc.) to the penned birds simultaneously with the pen environment applications.

If it becomes necessary to determine the major route of pesticide exposure to the test animals, then various combinations of treatments can be made. For example, treatments (with adequate replicates) might include (1) pen environment only with "clean" food and water, (2) "clean" pen environment and water with treated food only, (3) "clean" pen environment and food with treated water only, or (4) other combinations.

Birds in half the pens at a given treatment rate may be fasted and water withheld for 12 hours prior to the pesticide applications. If so, half the control pens should also be fasted and not allowed access to water fountains for the same 12-hour period.

All pens should be numbered and locations mapped or charted. Daily records should be kept of observations, toxic signs, test bird deaths and replacements (if any), complete data on pesticide formulations, application rates and methods, weather conditions and all other data of value in assessing the hazard to birds (see check list).

Reproductive success of the test birds may be determined. This should be done during the year of the test (and again 1 year post-treatment with survivors if residues of the test chemical are known to be persistent).

Eggs may be picked up periodically for artificial incubation and rearing of young in the first half of the breeding season but eggs may be left for the hen(s) to incubate in the last half of the normal breeding season. Hens should be allowed to rear the young to 14 days of age in the test pens.

The duration of the test should be a minimum of 21 days after the final pesticide application (longer if any birds are showing toxic signs or other effects). Reproductive tests would of course continue beyond 21 days posttreatment.

Pesticide residue determinations may be added to support the study throughout. Diet and water levels of the test chemicals should be confirmed. Vegetation, soil, and other environmental samples may be analyzed for residues in accordance with other label requirements and to determine persistence and bioaccumulation. Test birds poisoned by the pesticide and a sample of surviving birds should be analyzed for residues in selected tissues. Gross pathology may be determined at the same time.

Reporting of Data

The final report must include:

An introduction and background information.

Proposed use of the pesticide and related data such as vegetation or crop(s), formulation(s), and rate(s) of application.

Test Methods and Materials

Test animals and their history; Test pen description; Test animals—food and handling; Pesticide formulation(s) and application(s); Statistical methods; Weather data collection; Analytical methods for residue determinations; Clinical data methods; Reproductive study methods; Other methods utilized.

Results

Mortality—number, dates, etc.; Toxic signs; Weight changes; Food consumption; Clinical observations; Necropsy Observations; Residue analysis results; Weather data and inclusive dates of test; Reproduction test(s) results.

Summary and Conclusions

Potential hazards to wildlife should be identified in addition to toxicity results per se; Tables; References.

References

Black, C. T. and G. L. Zorb. 1965. Effect of malathion sprays on penned pheasants. Mich. Conserv. Dept. Research and Develop. Rpt. No. 34.

Heezen, K. L. 1973. Pesticide effects on pheasants. Job Complet. Rept. Proj. No. W-118-R-6, Job No. 119.1 Mich. Dept. Conserv. 21 p. (Mimeo)

Kreitzer, J. F. and J. W. Spann. 1968. Mortality among Bobwhites confined to a heptachlor contaminated environment. *J. Wildl. Manage.* 32(4):874-878.

Zorb, G. L. 1968. Effects of pesticides on wildlife. Job Complet. Rpt. Proj. No. W-118-R-1, Job No. 4. Mich. Dept. Conserv. 6 p. (Exhibit 2).

EXHIBIT 1

MICHIGAN DEPARTMENT OF CONSERVATION

(Research and Development Report No. 34 June 18, 1965)

EFFECT OF MALATHION SPRAYS ON PENNED PHEASANTS [1]

(By C. T. Black and G. L. Zorb)

Summary

Malathion in water solution, sprayed at many times the rates normally used in agriculture, apparently does not harm pheasants. Spraying of penned, nearly grown, young pheasants with malathion in water solution, at rates of one, five, and ten pounds per acre, did not produce any apparent harmful results. The following spring there was no apparent decline in egg production among any of the sprayed birds. Eggs from the sprayed birds showed normal hatchability, and chicks from these eggs survived normally to an age of three weeks, by which time abnormalities usually develop. An attempt to determine effect of malathion on pheasants, their eggs, and young, from nesting in 12 feet by 250 feet pens, failed because the pens were too small to permit normal nesting.

* * * * *

Introduction

The prospect of the use of the insecticides malathion and sevin to control the newly invading cereal leaf beetle in Southwestern Michigan in 1963 raised some important questions as to the impact of such a program on wildlife. While it was suspected that these chemicals would not be harmful, there was strong reason to suppose that both insecticides would be relatively safe. Still, there was more evidence at that time regarding sevin; not so, malathion. Consequently the Patuxent Wildlife Research Center of the Bureau of Sports Fisheries and Wildlife of the United States Department of Interior requested the Michigan Department of Conservation to make certain field observations in the spray area and to conduct experiments on confined birds.

This report presents observations on an experimental spraying of penned ring-necked pheasants with malathion. This study was financed through the Patuxent Wildlife Research Center. The pens and supervision were furnished by the Michigan Department of Conservation. Special thanks are due to Martin Pollok, the experienced, painstaking superintendent of the State Game Farm, without whose incessant care this project could not have been undertaken. Certain other phases were financed by Federal Aid in Wildlife Restoration Project W-40-R.

Specifically, it was our intent to subject pheasants in an artificial penned situation to as nearly the same spray they would have received in the wild—malathion in aqueous solution at a rate of one pound per acre. We also desired to subject them to sprays many times that rate to determine the effect to excessive rates such as might result from accident, or deliberate heavy application by an over-enthusiastic farmer. Also these heavy rates offer certain advantages in interpreting experimental results. For this purpose we selected rates of five and ten pounds per acre. The most artificial aspect, besides the penned environment, was the time of year when we were forced (by circumstances beyond our control) to undertake the experiment. Our birds were sprayed in mid-September; the normal time of cereal leaf beetle control is late April and May. However, we feel that there are certain indications from this experiment that malathion may be a relatively safe insecticide where the important game bird, the ring-necked pheasant, is concerned.

Methods

Pens; Placement of Birds. Sixteen new pens (12 feet by 150 feet) constructed at the State Game Farm at Mason, Michigan during the summer of 1963 alongside 20 pens already there made available a battery of 36 pens. Aluminum flashing between all pens 18 inches high and buried about six inches in the ground prevented run-off between pens and excessive fighting between cocks. Pheasants purchased from a private breeder were banded and released in the pens (one male and five females per pen, plus some surplus birds) on September 1, 1963. Eight birds died upon flying into the fence between that date and the application of spray. The surplus birds enabled keeping the pens fully stocked at the time of spraying, with the few extra birds split up among the control pens.

Experimental Design. The pheasants were subjected to different concentrations of malathion—one, five, and ten pounds per acre. This gave nine pens (nine males and 49 females) for each treatment and nine pens for a control. While ten pounds per acre is far above the general rate of field application this three-level application might help to interpret results. For example, if few or no deaths occurred under the ten pound treatment, a few possible deaths in the lower dosages could be blamed on other causes.

Pens for the various treatments were selected at random within blocks of four pens each. The 20 old pens had rank cover or quackgrass and weeds, while the cover in the 16 new pens had been clipped and trampled during pen construction so that there was much less natural cover. There was also some difference in the slope between portions of the pen area. The systematic selection of blocks prevented the possibility of all birds under one treatment falling in the same cover type of slope. Selection of treatments within blocks was done at random.

Application of Spray. The insecticide was applied with a new John Bean "Trojan" ten-gallon sprayer. After a little practice, the 12 feet by 150 feet area (1800 square feet) could be sprayed and the tank emptied in one trip.

Two polyethylene panels (six feet by twelve feet) held on each side of the pen and carried in adjoining pens, prevented drift during the application. In actual operation, even with some wind, holding the nozzle (on its 15 feet hose) close to the ground eliminated danger of drift. The aluminum flashing on the sides of each pen were sufficient to limit drift at the pen edge.

Malathion was applied on September 10. At each pen the water was poured from ten-gallon cans into the sprayer, the malathion added, the motor started to facilitate mixing. The sprayer was then taken to the far end of the pen to start the spray, and worked back to the gate. The pheasants all received some direct spray.

Water cans and food trays were removed from the pens before spraying. A supply of ear corn, enough to last a month, was placed in each pen and scattered in an area of short grass. Even the old pens had a trampled area where the aluminum flashing was installed, so that the corn was not protected by tall grass or weeds. The corn was not turned over during spraying; perhaps a third of the kernels on the under side of the ears was not directly sprayed. The corn received the spray in the same manner as the rest of the pen.

In a few cases where the timing was not exact and some spray remained when the end of the pen was reached, the tank was run out back into the pen. No extra solution was sprayed on the corn. It required the full day to spray all 27 pens. No birds were lost due to the extra disturbance of the sprayer and crew. Fresh water was provided after the spray was completed.

Autopsy Procedure. Dr. L. D. Fay, Game Section Pathologist, autopsied 36 hens from the experimental pens (9 hens from controls and 9 from each treatment) on September 25 and October 10 and 18, or 6, 21, and 29 days after spray, respectively. The hens to be autopsied were picked at random—first by pen, then by individual bird. Only one hen was taken from each pen, so once selected a pen was no longer available for choice.

Birds were killed by injecting 2-3 ml. of a solution of sodium pentobarbitol into the heart a few minutes before examination. Dr. R. K. Ringer, an avian physiologist from Michigan State University, collected certain endocrine glands for histological examination and observed the general autopsy. The control birds were examined first to observe the normal organs. Procedure was to (1) examine externally for lesions and mass parasitism; (2) skin out carcass and observe general condition of musculature; (3) remove breast to expose abdominal and thoracic organs in their natural position; (4) examine heart, lungs, air sac membranes, liver, spleen, intestine, ovary, and kidneys, for color, size, and pathologic lesions and other abnormalities; (5) split trachea, crop, gizzard and intestinal tract; wash and examine sediment grossly for parasites; and make smears of intestinal contents and examine under microscope; (6) remove liver and spleen intact from most birds and weigh, and take samples of liver, spleen and kidney for sectioning.

Handling of Eggs and Chicks. The experimental birds sprayed as described above were over-wintered in four pens containing corn shocks for shelter. The birds from each spray treatment were kept separated and in pens that had been sprayed with their particular treatment. Higher protein food was furnished to all birds in late winter. Eggs were collected from these birds between April 27 and May 7 and placed in an incubator on May 11. On May 18 the incubating eggs were candled and infertile eggs or those containing dead embryos were removed. The eggs hatched on June 1. All the eggs which failed to hatch were examined and cause of failure classified as (1) infertile, (2) embryo dead, noting approximate age at death, or (3) egg pipped but failed to hatch. The chicks were held for three weeks in 12 brooder houses with 16 to 27 chicks per house. There were three houses for chicks from each of the four treatments. Survival of chicks was noted, and their development and growth closely watched to see if any abnormalities resulted from the malathion.

Spraying Breeding Birds. After the egg collection was complete we put a male and female from the control lot (not sprayed with malathion the preceding fall) in each of 24 pens and sprayed alternate pens and food supply with malathion at the rate of 5 lb. per acre. These birds were then permitted to nest. However, excessive harassment by the males and injury to several hens resulted in our removing most males and rotating a few males through the pens. Regular observations were made as to the progress of nesting and the

[1] Contribution from Pittman-Robertson Project W-40-19, Michigan.

number of broods produced by the sprayed and normal birds.

Findings

Late Summer Spray on Young Birds—Immediate Effect. No birds died and there was no apparent change in the appearance or activity of any of the birds following the experimental spraying with malathion sprays. The autopsy of three hens from the control group and from each treatment on the 6th, 21st, and 29th day following the spray (12 birds each date) showed no differences between the controls and the birds exposed to malathion. Nor were there gross differences among the controls and the pheasants exposed to the different rates of malathion. Specifically, there were no gross pathological lesions attributable to malathion.

The liver and spleen were saved and sections mounted. Examination revealed no differences between controls and experimental birds. We also have slides of the brain, and pituitary, adrenals, thyroid and thymus glands. Examinations are not complete, but no indications of damage so far have been detected.

A few birds died in the pens during December. Two of these were found when fresh enough to autopsy. One had a broken neck and the other a broken leg. Birds were disturbed by wild cocks flying onto and from the tops of the pens, and apparently flew into pen wire with occasional fatal results.

Reproduction in Following Spring. Malathion appeared to have no effect on pheasant reproduction in experimental birds sprayed (birds, food and pen) at the rates of 1, 5, and 10 lb. of malathion per acre about eight months prior to the breeding season. Experimental birds fared as well as or better than the control birds in egg production, egg fertility, hatchability, and survival of chicks:

Treatment	Eggs collected per hen	Percent of infertile eggs	Percent of fertile eggs		
			Dead embryos	Chicks dying after hatching	Chicks surviving to 3 weeks
Control	2.1	9	20	11	69
1 lb/acre	2.8	7	30	1	68
5 lb/acre	2.4	2	22	8	71
10 lb/acre	2.6	2	11	2	84

Egg Production. There was no apparent decline in egg production in birds experimentally sprayed with malathion, when compared to the controls. Since egg collections were made from large pens that contained shocked corn for winter cover, some eggs could have been missed. Each pen contained about 36 hens and the collection figures give at least an indication of production.

Treatment	Number of hens	Number of eggs	Eggs per hen
Control	37	77	2.1
1 lb/acre	36	102	2.8
5 lb/acre	34	81	2.4
10 lb/acre	35	90	2.6

Egg Fertility and Dead Embryos. The eggs were candled on May 18, one week after incubation began, and the infertile eggs and those with dead embryos were removed. At this time the eggs from the control birds and those from the 1 lb. treatment of malathion had greater losses than the 5 and 10 lb. groups.

On June 1, when most eggs hatched, the eggs which failed to hatch were examined and the dead embryos were aged. Embryos from the 1 and 5 lb. groups had suffered the greatest loss:

Treatment	Stage of Incubation					Total loss from all eggs	
	At 7th day			8th to 22d day		Number	Percent
	Number of eggs	Infertile	Dead embryos	Dead embryos	Pipped but died		
Control	77	7	6	4	4	21	27
1 lb/acre	102	7	7	15	4	36	35
5 lb/acre	81	2	2	13	2	19	23
10 lb/acre	90	2	3	6	2	13	14

Embryo loss was greatest in the 1 lb. experimental birds, and all experimental birds suffered heavier losses at the later stages of incubation than did controls. However, the eggs from the 10 lb. birds had the highest fertility rate and the lowest loss of embryos; this would seem to preclude damage from malathion.

	Age of dead embryos in days						Pipped but died	Loss of fertile eggs	
	1 to 7	8 to 10	11 to 13	14 to 16	17 to 19	20 to 22		Number	percent
Control	6	2	0	0	1	1	4	14	20
1 lb/acre	7	4	2	1	5	6	4	29	30
5 lb/acre	2	0	3	2	3	5	2	17	22
10 lb/acre	3	0	0	0	0	6	2	11	13

Survival of Chicks. Chicks from each treatment were placed in brooder houses (from 18 to 27 chicks per house) and raised to an age of 3 weeks. The controls suffered heavier losses than did experimental birds. Our game farm manager noted nothing unusual in the losses that occurred. Loss was from picking, occasional runts, and a few deformed birds.

Treatment	Pen No.	Number of chicks	Total chicks in treatment	Died to 8 weeks		
				Number per pen	Total	Percent
Control	7	19	55	4	8	14
	8	18		2		
	10	19		2		
1 lb/acre	4	22	66	0	1	2
	5	22		0		
	6	22		1		
5 lb/acre	11	22	62	2	6	10
	12	21		3		
	14	10		1		
10 lb/acre	1	27	77	1	2	3
	2	25		1		
	3	25		0		
All experimental Birds			205		8	4

Spring Spray on Breeding Birds. We were unable to evaluate the effects of a 5 lb. per acre application of malathion applied to birds during the breeding season. Nesting was not succesful in the 24 pens each containing a pair of birds. The cock harrassment of the hen was a major problem. Most of the cocks were removed and the remaining ones alternated between pens so that the hens would have an opportunity to begin nesting but still insure fertility. Nine hens died from accidents or from injuries from the cock, nine hens raised no brood, and six hens hatched clutches. The six successful clutches produced only 12 chicks.

EXHIBIT 2

JOB COMPLETION REPORT

RESEARCH PROJECT SEGMENT

State of Michigan
Project No.: W-118-R-1
Job No.: 4
Period Covered: July 1, 1967, to June 30, 1968
Name: Farm Game Management Research
Title: Effects of Pesticides on Wildlife

Abstracts. Simulated field applications were made of phorate and phosphamidon on ring-necked pheasants confined in .04 acre pens (12 ft x 150 ft). The phorate granules were spread evenly over the entire pen area, including the food. Phosphamidon was applied evenly in a 10-gallon water mixture over the entire pen including the food supply. We made no attempts to spray or to avoid spraying the pheasants. Many of the birds received some direct spray but none were heavily sprayed. This is the same procedure we have followed in our tests of five other organophosphate insecticides.

Phorate was applied twice, three weeks apart, at ½ and 3 pounds per acre rates. Phosphamidon at ½ pound per acre was applied twice (a month between applications), and applied only once at a 3 pound per acre rate. Surviving birds were held over winter and eggs collected and hatched, and the chicks held for two weeks to check reproductive success.

Under our experimental procedures phorate was not dangerous to pheasants. One bird dead four days after the initial application of phorate at 3 pounds per acre, was the only bird to show any ill effects out of the 60 experimental birds subjected to the two applications of this granular pesticide. No adverse effects on reproductive success were discernable in the treated birds the following spring.

Phosphamidon at a rate of 3 pounds per acre had serious effects on the birds. Nine birds died and 16 of the remaining 21 birds became immobilized under the 3 pound rate. The sick birds probably would not have survived under natural conditions resulting in a possible 90 percent mortality from this rate of phosphamidon. After two applications of phosphamidon at ½ pound per acre, four birds became sick after the first application, but there were no deaths and no other ill effects.

The surviving birds from both treatments had the same reproductive success as the control birds when checked the following spring.

Modified spray treatments. As a further check on just how the birds in our pens were receiving an organo-phosphate pesticide, we sprayed diazinon at a rate of 5 pounds per acre in three modifications of exposure of the pesticide to the birds. In three pens only the food was sprayed, in three pens only the birds were sprayed and in three pens the birds and food were introduced immediately after the entire pen was sprayed. Two pens were sprayed with the birds and food in the pen, following our standard spray procedure.

There was almost complete mortality in the "food only", "birds only", and the "normal" spray applications. Where only the environment was sprayed there was no effect at all on the birds. Either contaminated food or direct spray killed the birds but a freshly sprayed environment had no effect. Apparently the diazinon must be oral, dermal or respiratory, rather than through casual exposure. The 5 pound rate of diazinon sprayed pens with birds and food in them had much more effects on the pheasants than our tests, conducted in the same manner two years ago. This year we had 100 percent mortality, compared to 40 percent mortality in the previous test. We have no explanation for this difference. It would be beneficial if we could set up future experiments with modified spray treatments to indicate more clearly the manner of transmission of the pesticide to the birds.

Recommendations. Continue to test nonpersistent pesticides on penned pheasants. Make selection of test materials after discussion with State and Federal authorities so that the work will be done with the chemicals that are the most important to Michigan or to the pesticide situation as a whole. Work toward a testing procedure that can be utilized as a standard of "simulated field tests" everywhere in the country.

Objectives. To study the effects of insecticides on game birds and mammals and all other wildlife under field and pen conditions.

Techniques used. During August and September of 1967 we checked the effects of phorate (Thimet) and phosphamidon on pheasants under simulated field conditions. Phorate (10 percent granular on attapulgite) was spread on .04-acre pens with a hand windmill seeder at the rates of ½ and 3 pounds per acre. Phosphamidon, in a 10-gallon water mixture, was sprayed at these same rates. There were five pens, each with six birds (1M 5F), for each treatment plus five pens of control birds. The birds received no direct contact with phorate, but it was spread on and around their food supply and over the entire area of the pen. In order to assure even coverage with the phorate we added additional attapulgite of a similar mesh size

for filler. With the liquid mixture of phosphamidon we made no attempt to spray, or to avoid spraying the birds directly. A few birds in each pen received some direct spray. Phorate was applied twice (August 5 and 28). Phosphamidon was applied twice at the ½-pound rate (Aug. 8 and Sept. 6) and once (Aug. 8) at the 3-pound rate.

In an attempt to clarify just how birds receive pesticides under our spray conditions we set aside 11 pens for different application methods. Using diazinon at a rate of 5 pounds per acre we sprayed (1) only the food supply in three pens, (2) only the birds in the three pens, (3) the pen only, omitting food, water and birds in three pens, and (4) the remaining two pens in the normal manner.

Spray Results—Phorate. Under the experimental conditions, phorate had little effect on penned pheasants. One bird died four days after the first application of phorate at the 3-pound rate. There were no other deaths and the remainder of the birds appeared normal at all times.

Treatment	Number of pens	Birds	Died	Sick	Number of applications
Phorate ½ lb	5	30	0	0	2
Phorate 3 lb	5	30	1	0	2

The pens had a heavy grass cover when the granular phorate was applied. A mowed strip 150 ft. long down the center of each pen, and relatively bare areas around the feeder, at both ends of the pen, and along the fence provided areas where the granular particles would be readily available to the birds.

Phosphamidon. Phosphamidon at a rate of ½ pound per acre had no serious effects on pheasants but when the rate was doubled to 3 pounds per acre, 90 percent of the birds were killed or become immobilized for such long periods that it is doubtful that they would have survived under natural conditions. No birds died and three became sick after the first application of phosphamidon at the ½ lb. per acre rate. A second application was made a month later and at this time only one bird became sick and none died.

The 3-pound rate had more pronounced effects on the pheasants. Nine birds died over a 10-day period and 18 of the remaining 21 birds became sick. The sick birds became immobilized, had a watery discharge from their bills, and were unable to eat. Four of the dead birds and several of the sick birds had badly scalped heads. We didn't observe how the scalping occurred, and we had never observed this on any of the sick or dead birds from other organo-phosphate insecticide trials we have completed. The sick birds would have had a difficult time surviving under natural conditions.

Treatment	Number of pens	Birds	Died	Sick	Number of applications
Phosphamidon ½ lb	5	30	0	4	2
Phosphamidon 3 lb	5	30	9	18	1

Diazinon. In our original tests with diazinon in 1966 we lost 17 of the 42 experimental birds under the 5-pound per acre treatment. This year in two pens, treated exactly as in the original experiment, we lost all 12 of the test birds. The diazinon was newly purchased, from fresh stock according to the vendor, and we have no explanation for the increased vulnerability of the birds this year. For the food treatment we spread the cracked grain on burlap sacks, sprayed it and returned it in food trays to the pens. For the "birds only" treatment, the birds were placed in small cages and submitted to direct spray for 4 to 5 seconds. For the "pen only" treatment the food, water and birds were removed from the pen before the spray application and put back into the pen immediately after the pen was sprayed.

The results of diazinon at a rate of 5 lb. per acre on pheasants under the different conditions were as follows:

Treatment	Birds exposed	Results	
		Sick	Died
Food only	18	2	15
Birds only	18	0	17
Pen only	18	0	0
Complete spray	12	0	12

The birds were affected either through the contaminated food or by direct spray, receiving the diazinon via either oral, dermal, or respiratory routes. Contact with a newly sprayed environment did not affect the birds.

The phorate and phosphamidon survivors were placed in separate pens according to original treatment and held over winter. They were returned to their original pens in April, and eggs were collected for reproduction trials.

Hatching results. There was no indication that the experimental birds differed from control birds in their reproductive success. The data from one pen of birds from the phosphamidon (½ pound rate) treatment were omitted from the analysis. The cock in this pen was apparently infertile as none of the 57 eggs collected from this pen were fertile. To check this we put a different cock in this pen and of 20 eggs collected after the change, 18 were fertile. The basic hatching data is given in Tables I and II.

An analysis of the variance of the number of chicks surviving to two weeks per egg laid, for the controls and the four treatments is shown in Table III.

Prepared by Gordon Zorb,
Approved by ————————
Date October 7, 1968.
Approved by Ralph J. ————————.

TABLE III.—*2-week chicks per egg laid*

Pen No.	Controls	Phorate ½ lb	Phorate 3 lb	Phosphamidon ½ lb	Phosphamidon
1	0.66	0.79	0.64	0.61	0.81
2	.80	.70	.59	.85	.69
3	.81	.68	.79	.65	.86
4	.45	.86	.64	.67	.89
5	.68	.71	.67		.53
Average	.68	.75	.67	.70	.76
Total	3.40	3.74	3.33	2.78	3.79

NOTE.—See table below:

Source	df	S.S.	M.S.
Among treatments	4	0.034	0.0085
Within treatments	19	.250	.0132
Total	23	.284	

$F=0.644$:

$F_{.95}(4, 19)=2.90$

PROTOCOL FOR FULL-SCALE FIELD TESTS TO EVALUATE PESTICIDE HAZARDS TO WILDLIFE

No single test protocol can be designed to cover all field studies because practical considerations for each field situation and compound make each study unique. It may be possible to develop similar protocols for a uniform type habitat in a given geographical region (example—study of grasshopper insecticides in short-grass plains habitat—McEwen *et al.* 1972).

The primary objective of a full-scale field study is to determine the total impact of pesticide applications on wildlife populations. Effects may occur in many ways including (1) direct poisoning and death (by ingestion, dermal exposure, or inhalation), (2) toxic effects indirectly causing death such as those increasing susceptibility to capture by a predator, (3) causing inability to reach food and water or lower resistance to weather and disease stresses, (4) loss of insect food supply and abandonment of home range, (5) harmful reproductive effects and consequent inability to maintain populations, or (6) other indirect ecological disturbances.

Full-scale field tests are applicable for proposed pesticide uses to be registered for large-scale spraying on forest, rangelands, rights-of-way, roadsides, wetlands, and major croplands (such as cotton, wheat, corn, soybeans, rice, sorghums, or alfalfa) or other major wildlife habitats. Data available from other tests such as those to determine efficacy, persistence, environmental contamination, transfer and bioaccumulation potential, and residues in livestock, may be useful in developing an estimate of total ecological effects. Such data may be nearly sufficient or may need much expansion depending on their completeness.

METHODS AND MATERIALS

A thorough pesticide-wildlife ecology study should include collection of data on wild bird and mammal populations (resident and nonresident species), climate, soil, vegetation biomass by species, numbers and biomass of arthropods (identified to lowest taxonomic identification possible), food habits of the most abundant wildlife species, and distribution and fate of pesticide residues in animals, plants, and environment. Each parameter or variable would require a separate sampling method. These data should be collected on sprayed and unsprayed areas before and after the treatment date(s).

The experimental pesticide applications should be made on the crop(s) or type of vegetation for which the pesticide will be registered. Wildland treatment areas should be a minimum of 129.5 ha (320 acres) in size for a given chemical and rate of application. Cropland or right-of-way study areas may be smaller if the typical field or area sprayed would be smaller. All treatment areas should be sufficiently large to accommodate a minimum of 2 replicates of 8.1 to 16.2 ha (20 to 40 acres) census plots with a sprayed buffer zone of at least 45.7 m (150 ft) around all plot boundaries. The experimental applications should be made at the proposed registration rate and at 2 multiples of that rate (such as 3 or 5 x and 5 or 10 x). Unsprayed cropland or wildland habitat should be studied simultaneously on replicated plots in the same manner as the sprayed areas in order to detect changes due to factors other than the pesticides.

Cropland or rangeland birds may be censused by adaptations of the *strip method* (Emlen 1971; McEwen *et al.* 1972). Emphasis should be on spring and summer resident species during the breeding season. Birds in forested or mixed habitats are usually censused by a *plot method* (Ecological Research Committee 1970).

The basic procedure in the *strip census* technique is to walk a straight line transect, usually within a given time period, and to identify, record, and plot locations of all birds seen within a predetermined width of the strip (for example, 50 m to either side of the line of travel, i.e., a width of 100 m). Strip censuses are usually run in the early morning hours to coincide with a major activity period of the birds. Transects are marked in some manner so that the same routes can be repeated periodically over several days, weeks, months, or from year to year. Frequent counts within the breeding season on replicated transects will provide statistically adequate data for comparing pre- and postspray populations and sprayed plots with unsprayed plots.

The general approach for *plot censuses* in woody or mixed habitats is similar to the strip census. The basic difference is that birds are observed, identified and plotted on a map of a rectangular or square plot (usually about 16 ha or 40 acres in size). The observer walks a more or less set route taking him to all portions of the census plot within a given time period. Other requirements such as season, time of day, replications, frequency of repetition, etc., are similar to the strip method.

With either method, weather conditions must be suitable for the counts. All nests found should be marked and checked periodically with a minimum of disturbance to determine nesting success. Reproductive success may also be estimated by recording the number of fledglings observed per unit of area and time on replicated plots.

Small mammals may be studied by a mark and recapture method using grids of Sherman-type live traps. There are various systems of trap layouts, length of trapping period, and data treatment. The system recommended by the International Biological Program (IBP), Grassland Biome (Swift and French 1972), utilized a square grid of 12 x 12 stations or 144 trap sites (15 m between stations) with 1 or 2 live traps per station. Animals captured are marked and released over a trapping period of 5 consecutive days. The same procedure is repeated in a later time period(s) (example, pre- and postspray) on marked, replicated grids. Data are analyzed by the Jolly (1965) method.

Effects of the pesticide treatments on total arthropod numbers and biomass should be measured in addition to effects on the target insect. These measurements can be made by standard entomological methods. Particular attention should be paid to arthropod species known to be important for wildlife food. The limitations of the arthropod sampling techniques used should be noted and reported.

Animal and environmental samples should be collected for pesticide residue determinations periodically until residue levels fall below 0.01 ppm. The following types of samples should be analyzed: (1) Tissues of 1 or more species of common resident omnivorous birds, (2) tissues of 1 or more species of common resident omnivorous mammals, (3) common arthropods, (4) vegetation (leaves and stems or entire above-ground parts of the wild or cultivated plants sprayed), (5) plant litter, (6) soil (to a depth of about 2½ cm), and (7) water (if any) from the sprayed area. Much of the pesticide residue data may also be required for other phases of the registration procedure and may be utilized for dual purposes.

REPORTING OF DATA

The final report should include:

An introduction and background information

Proposed use of the pesticide and related data such as vegetation or crop(s), formulation(s), and rate(s) of application.

Test methods and materials

A description of the study area including vegetation, topography and all pertinent ecological information; Study plot layout—locations and replications; Listing of resident and migrant fauna with estimates of population densities or relative abundance; Pesticide formulation(s) and application(s) including details of spraying equipment, methods, and weather conditions.

Statistical design and methods of analysis; Weather data collection; Analytical methods for residue determinations; Clinical data methods; Reproductive study methods; Carcass search methods; Other methods utilized.

Results

Mortality—number, dates, etc.; Signs of intoxication; Bird and mammal census results; Arthropod numbers and biomass; Food habits data, if any; Necropsy observations; Residue analysis results; Weather data and inclusive dates of test; Results of nest studies, fledgling observations, and other studies of effects on reproduction of resident wildlife; Integration of all data into a picture of the total ecology of the introduction of the pesticide.

Summary and Conclusions

Potential hazards to wildlife should be estimated with full consideration of possible indirect effects due to ecological disturbances.

Tables

References

References

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EXHIBIT 1

VERTEBRATES—SMALL MAMMALS

Sampling Methods—Live Trapping

Small mammal populations will be sampled by marking, releasing, and recapturing animals in grids of live traps. Where possible a grid should be established and run on each grazing treatment. The grids will consist of 12 x 12 stations, with two traps per station. Stations will be 15 m apart. Each grid will cover an area of 6.8 acres (2.7 ha). There will be at least five consecutive days of trap-mark-release on the grids. This will be followed by five consecutive days of trap-mark-release on assessment lines. This will provide basic data for estimation of population densities. Density of major species will be estimated four times during the year at the southern sites (Jornada and Pantex) and three times at the other sites.

Rodents will be marked by a system of toe amputation and/or with numbered metal ear tags. Fig. 1 shows the method to be used. Looking at the underside of the animal, the feet are read from left to right and top to bottom to give the four digits of the identification number, one digit per foot. The toes on each foot are read from left to right. If there are four toes on the foot, then there are four possible numbers that can be assigned to that particular foot: 1, 2, 3, or 4. If there are five toes on the foot, there are five possible numbers. Clipping of one toe from each foot gives a four-digit identification number. Animals with toes like the illustration will provide a possible 899 different combinations of four-digit numbers to be assigned to that species. This requires clipping of no more than one toe per foot. In the case of a natural amputation which occurs after marking, there will be two toes missing from the foot. In such cases (provided that the natural amputation cannot be distinguished from the artificial), that digit of the identification number is indicated as a 9.

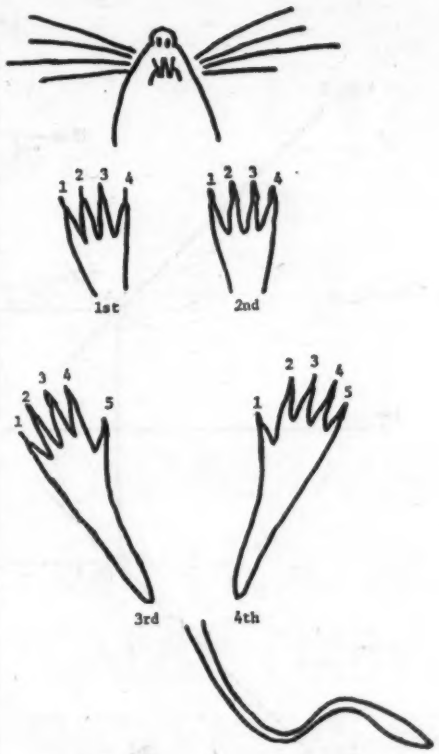
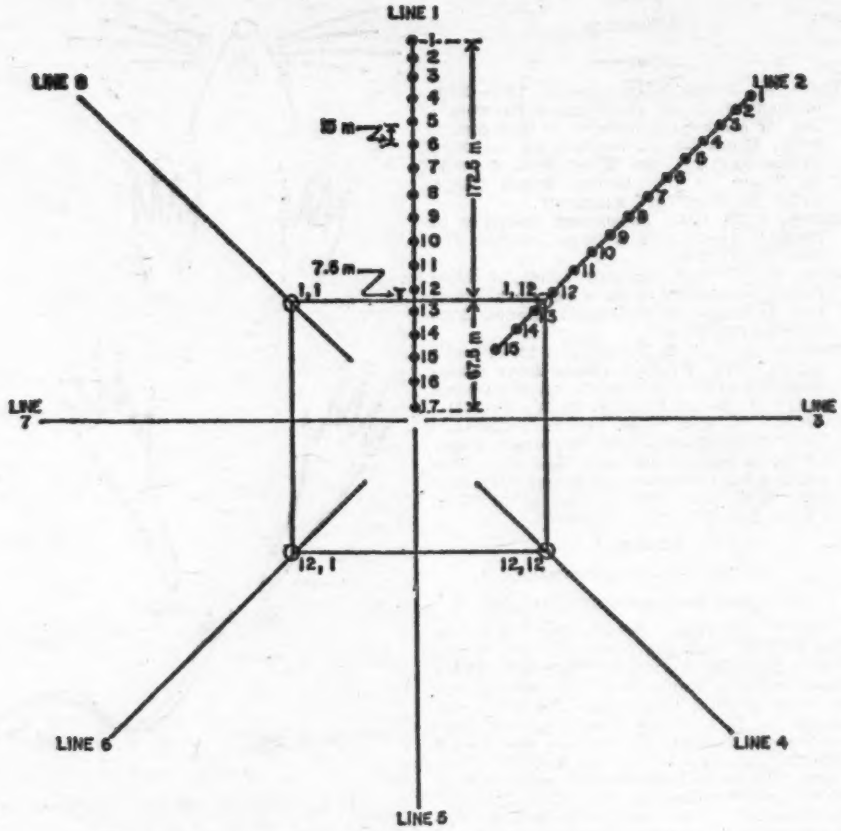


Fig. 1. Four-digit number for rodent marking.

This system often permits the original identity of the animal to be determined without confusion.

Trapping on assessment lines will permit estimation of the area sampled by the grid. Animals captured on the assessment lines are examined to see if they were marked on the grid. Unmarked animals will *not* be marked. All animals trapped will be released. Interstation interval on these lines will be 15 m with two traps per station. Eight assessment lines will be established per grid. Four of the lines will bisect the corner angles of the grid and will extend 37.5 m into the grid. The other four lines will be perpendicular bisectors of the grid sides and will extend 67.5 m into the grid. All eight lines should extend out 172.5 m from the grid. Diagonal lines will thus have 15 sets each, and perpendicular lines 17 sets each—for a total of 256 traps (two traps per set). The assessment line which bisects Row 1 of the grid will be Line 1, and the other lines will be numbered sequentially in a clockwise direction. Trap stations on each line will be numbered sequentially with the outermost trap on each line being Trap 1. Fig. 2 shows the design of the grid and assessment lines.



$\bullet_n$ Trap stations on assessment lines (shown only for lines 1 and 2).

$O_{n,n}$ Grid stations (only corner stations shown).

Fig. 2. Small mammal trap grid and assessment lines (not drawn to scale).

SAMPLING METHODS—SNAP TRAPPING

Kill trapping will be conducted to obtain information on diets. In the process, some demographic data will be derived from these specimens. Animals trapped will be removed and saved for autopsy. At autopsy the animals will be weighed and measured; the testes of the males will be measured to the nearest millimeter, both length and width. For females, the condition of the mammary glands and the reproductive tracts will be recorded. *Nipples* may be small (non-lactating, nulliparous females); enlarged but not prominent (non-lactating, parous females); or enlarged and prominent (lactating, parous females). The *total number of embryos* will be recorded, including those that appear to be abnormal or resorbing. The number being *resorbed* will be recorded separately and will include only those embryos which are distinctly smaller and undergoing tissue lysis and disorganization. The *embryo length* will be recorded as the crown-rump length in millimeters of the embryo as it lies in its normal position. The total weight of the embryos and reproductive tract combined will be recorded to the nearest 0.01 g. Numbers of new and old *placental scars* will be recorded. New scars are distinguished by plentiful blood supply to the implantation site and/or a large amount of dark pigment deposited at the site. Old scars are distinguished by lack of blood supply and fainter pigmentation. The number of *corpora lutea* can be determined from the intact ovaries by examining the surface of the ovaries for firm, spheroidal, pinkish to yellowish structures.

Total stomach contents of all individuals of each major species will be preserved in Formalin and labeled. The label should contain the following information: i. site, ii. date collected, iii. collector's number, iv. code for genus and species, v. sex, and vi. age (adult or juvenile).

SAMPLING METHODS—POCKET GOPHERS

Pocket gophers will be censued by a mark-release-recapture method. Live traps will be set in exposed burrows where animal activity is evident.

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